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Screening, Selection and Production of Extracellular Methanol and Ethanol Tolerant Lipase from *Acinetobacter* sp. K5b4

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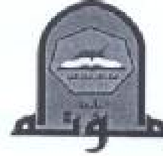
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الآراء الواردة في الرسالة الجامعية لا تُعبر

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DEDICATION

My heartfelt thanks and deepest appreciation to my husband Muwafaq for his continuous support and understanding until the end and for my wonderful daughters Lojain, Aram and Rowa' for their continuous innocent prays.

I will not forget my parents who encouraged me to complete my study since childhood and many thanks to all who gave me support, encouragement and motivation.

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Abstract

Screening, Selection and Production of Extracellular Methanol and Ethanol Tolerant Lipase from *Acinetobacter* sp. K5b4

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A total of 34 of bacterial isolates with extracellular lipolytic activity were isolated from hydrocarbons contaminated soil samples collected from 7 different petrol stations and car repair shops in Al-karark, south of Jordan. Seven of these isolates were able to retain100% of their original activity after exposure to methanol and ethanol organic solvents. Among the seven isolates and based on its relative activity, the lipase produced by the bacterial isolate K5b4 exhibited the highest stability in presence of different concentrations of methanol and ethanol in the assay solution and was selected for further studies.

The identification of the K5b4 bacterial isolate using the RapID™ ONE System and 16S rRNA gene sequencing were close in identity to *Acinetobacter* sp. The optimization of the physiochemical parameters of the growth conditions resulted in maximum lipase production of 31.5 mU/mL when the optimized culture media (pH 7.0) inoculated with 0.4% (v/v) inoculums size and incubated at 30 ^{o}C and 150 rpm agitation speed.

The optimum chemical composition of the growth medium supported the highest enzyme production was 1.0% (w/v) olive oil, 0.2% (w/v) glycerol and 0.15% (w/v) yeast extract. Inclusion of different mineral salts in the culture media were did not enhance the enzyme production as it compared to the control experiment. Meanwhile, the enzyme and biomass production was increased with the increase in NaCl concentration with the maximum enzyme and biomass production obtained when 0.05% (w/v) of NaCl was added to the growth media.

The inhibition of lipase production from *Acinetobacter* sp. K5b4 in presence of glucose suggesting that lipase gene expression is prone to catabolic repression.

الملخص

فحص وأختيار وإنتاج لايبيز خارج خلوي قادر على تحمل الميثانول والإيثانول من *Acinetobacter* sp. K5b4

ميساء راتب العنانزة

جامعة مؤتة ، 2014

في هذه الدراسة تم عزل ما مجموعه 34 سلالة من البكتيريا التي تمتاز بنشاطها في إنتاج محللات الدهون خارج الخلية (انزيم اللايبيز)، من تربة ملوثة بمواد هيدروكربونية من 7 محطات بنزين ومحلات تصليح سيارات مختلفة في محافظة الكرك جنوب الأردن.

سبعة من هذه العزلات كانت قادرة على استعادة 100% من نشاطها الأصلي بعد تعرضها إلى تراكيز مختلفه من المذيبات العضوية الايثانول والميثانول، ومن بين العزلات السبعة تم اختيار سلالة البكتيريا K5b4 بناءا على النشاط النسبي لأنزيم اللايبيز المنتج من قبل هذه السلاله فقد كان انزيم اللايبيز المنتج من قبل هذه العزله من اكثر الانزيمات استقرارا بوجود تراكيز مختلفة من الايثانول والميثانول في محاليل الفحص.

وقد تم تعريف عزلة البكتيريا باستخدام أدوات التعريف البيوكيميائية والتسلسل الجيني عن طريق 16S rRNA والتي دلت الى أن البكتيريا هي من نوع *Acinetobacter*، وتلا تعريف العزله تحديد العوامل الفيزيائية والتركيب الكيميائي الامثل للوسط الغذائي والذي نتج عنه ما يقارب ال 31.5 mU/ml. و كانت الظروف الفيزيائيه المثلى كما يلي: 0.4%(v/v) لحجم العينة المزروعة، 30 درجة مئوية و 150 rpm لحضانة البكتيريا، ودرجة حموضة للوسط الغذائي 7.0.

أما التركيب الكيميائي الأمثل الذي حفز اعلى مقدار إنتاج من انزيم اللايبيز فقد كان بأستخدم زيت الزيتون كعامل محفز بتركيز (1.0% w/v)، الجليسيرول كمصدر كربوني بتركيز (0.2% w/v) و مستخلص الخميرة كمصدر نيتروجيني بتركيز (0.15%w/v). دلت النتائج ايضا الى ان أضافة انواع مختلفه من املاح المعادن الى الوسط الغذائي لم تزيد من انتاج الانزيم مقارنه مع الوسط الغذائي بدون وجود الاملاح المعدنيه في حين ان اضافة ملح كلوريد الصوديوم الى الوسط الغذائي بتركيز 0.05% (w/v) قد ادى الى زيادة انتاج الانزيم من البكتيريا المختاره.

ولقد لوحظ إن وجود الجلوكوز في الوسط الغذائي يقلل من إنتاج اللاييز بسبب تأثيره السلبي على الجين المنتج للايبيز في سلالة K5b4 لذا اعتبر عامل مثبط لإفراز اللايبيز .

CHAPTER ONE

Theoretical Background

1.1 The study background.

Enzymes are novel biocatalysts; represent the key tool in industrial biotechnology and always contributes to clean industrial products and processes (Drepper *et al*., 2006). Compared to chemicals, enzymes are specific in their action, selective to their substrate, often carry out reactions which are not even possible with conventional chemistry and they are compatible with the environment (Schäfer *et al*., 2007). Among the most important biocatalysts carrying out novel reactions in both aqueous and non aqueous media, lipases stand out due to their versatility, regio and enantioselectivity, wide spectrum of substrates specificity, high stability towards extreme temperatures, pH and organic solvents (Freitas *et al*., 2007).

Lipases (carboxyl ester hydrolases E.C. 3.1.1.3) are water-soluble enzymes that catalyze the hydrolysis of carboxyl ester bonds in triacylglycerols at the oil-water interface (Cygler and Schrag, 1997)**,** and more specifically defined as long-chain fatty acid ester hydrolases, with "long-chain fatty acid" meaning aliphatic acids, saturated or unsaturated, with twelve or more carbon atoms with glycerol as alcohol moiety. Lipases may be isolated from animals, plants and microorganisms. Microbial lipases are commercially important industrial enzymes due to their great diversity (Alves *et al*., 2002) and their potential for large scale production (Hasanuzzaman *et al*., 2004). A considerable number of lipases produced by bacteria, yeast and fungi have been isolated (Joseph *et al*., 2008). The extracellular bacterial lipases are of considerable commercial importance as their bulk production is more economical (Snellman *et al*., 2002). Some important lipase-producing bacterial genera include *Bacillus*, *Burkholderia* and *Pseudomonas* (Gupta *et al*., 2004) which are widely used for a variety of biotechnological applications (Beisson *et al*. 2000). Bacterial lipases are mostly extracellular (Gupta *et al*., 2004) and constitutive in their expression (Sharma *et al*., 2011). Medium composition and several physiochemical factors such as temperature, pH, and agitation have been always reported to be greatly influence the production of extracellular microbial lipases (Veerapagu *et al*., 2013). In addition Lipases are inducible enzymes where their expression is greatly affected by the presence of their natural lipid inducers(Veerapagu *et al*., 2013). Other components in the culture media such as carbon source (other than lipids), nitrogen source and essential micronutrients have been shown to be of great

important and carefully must be considered for maximum enzyme production (Veerapagu *et al*., 2013; Gupta *et al*., 2004).

Lipases constitute a very important group of biocatalysts for biotechnological applications in the detergent formulation (Saisubramanian *et al*., 2006), food processing (Olempska-Beer *et al*., 2006), flavour esters synthesis (De los Ríos *et al*., 2008), biocatalytic resolution of pharmaceuticals (Yadav and Dhoot, 2009), bioactive fatty amide derivative synthesis (Khare *et al*., 2009), as biosensor, bioremediation of hydrocarbons (Gaur *et al*., 2008), cosmetics industry (Yadav and Dhoot, 2009), perfumery (Fujiwara *et al*., 2006), and in biodiesel production (Shah and Gupta, 2007), etc. One of the stunning applications of lipases is their use as biocatalyst in the enzymatic transesterification reaction between oil and alcohols for the production of biodiesel. Due to the global shortages of fossil fuels, significant increase in the price of crude oil and increased environmental concerns (Fan *et al*., 2012) biodiesel have been immerged as an alternative energy source to conventional fuel (Narwal and Gupta, 2013) with zero carbon dioxide emission if pure biodiesel have been used (Vasudevan and Briggs, 2008). Biodiesel is a mixture of fatty acid alcohol esters (Ghaly *et al*., 2010). Biodiesel is generally produced through transesterification reaction catalyzed either chemically or enzymatically (Fan *et al*., 2012) . In the production of biodiesel using the enzymatic transesterification , the most frequently used alcohols includes: methanol and ethanol (Ghaly *et al*., 2010) Therefore, for lipase enzyme to be used as the biocatalyst in the transeterification reaction, they must be stable in the presence of different alcohols including the most frequently used methanol and ethanol.

1.2 Aims of the study.

1- Isolation and selection of extracellular lipase producing bacteria from soil samples.
2- Selection of methanol and ethanol tolerant lipase producing bacteria.
3- Optimization of the growth medium composition as well as the physicochemical factors that result in maximum enzyme production.

CHAPTER TWO
Review of Literature

2.1 Lipase enzyme.

Lipases (Triacylglycerol hydrolases EC 3.1.1.3) are enzymes which are capable of hydrolyzing triglycerides, diglycerides and monoglycerides into fatty acids and glycerol. According to the classification recommended by the enzyme commission of the international union of biochemists, lipases have been numbered as EC 3.1.1.3 which mean it’s belong to hydrolytic enzymes (class 3) acting on ester bonds (subclass 1) of carboxylic acids (sub-sub-class 1) of triacylglycerols (3). Lipases belong to the class of serine hydrolases and usually do not require any cofactor (Gupta *et al*., 2004). The structural characterization of the enzyme active site showed that the catalytic triad is composed of Ser- His-Asp/Glu (Gupta *et al*., 2004)

The natural substrates of lipases are long-chain triacylglycerols, which have very low solubility in water. Therefore, lipases can hydrolyze fats to fatty acids and glycerol at the water-lipid interface. Lipases may exist in two different conformations, an open active conformation and a closed inactive conformation. In an aqueous homogeneous media, these forms are in equilibrium shifted towards the closed form while in the presence of hydrophobic surfaces the equilibrium shifted towards the open conformation (Lan *et al*., 2011). The active site shielded by mobile lid, whose position closed or open determines the enzyme in an inactive or active conformation. If the enzyme is active the lid will be open and the substrate will access to the active site (Joseph *et al*., 2008). Based on their regiospecificity, lipases can be divided into two groups, first group catalyses the complete breakdown of triacylglycerol to glycerol and free fatty acids together with diacylglycerols and monoacylglycerols as intermediates in the reaction. The second group release fatty acids from outer 1 and 3 positions of acylglycerols to give free fatty acids, 1,2- diacylglycerol, 2, 3 and 2- monoacylglycerol (Patil *et al*., 2011).

Lipases are naturally used by organisms such as plants, animals and microorganisms as hydrolytic enzymes in their own lipid metabolic pathways (Patil *et al*., 2011; Pahoga and Sethar, 2002). Lipases are able to catalyze various reactions in aqueous solutions and organic solvents such as hydrolysis, esterification, interesterification, acidolysis, alcoholysis and aminolysis (Sharma and Kanwar, 2014). The direction of the reaction depends upon the water content available. In high water media the reaction is

reversible, and lipases catalyse hydrolysis, alcholysis, aminolysis and acidolysis. But in low water media the reaction is irreversible so that lipases catalyse esterification, transesterification and interesterification (Vakhlu and Kour, 2006).

Several lipases have been reported in the literature with great stability characteristics against temperature and pH. The main advantage of these enzymes is the stability in organic solvents which lead to the designing of novel drugs, surfactants, bioactive compounds and oleochemicals (Ankit *et al*., 2011; Gupta *et al*., 2004; Ghosh *et al*., 1996).The ability of lipase enzyme to retain its activity in the presence of organic solvents is an attractive property, as many reaction media for enzymatic reactions involve the use of organic solvents (Sharma and Kanwar, 2014). In an organic solvent environment it is possible to conduct reactions that are suppressed in water environments (Rahman *et al*., 2006). Besides their stability in organic solvents, the potential biotechnological applications of lipases is due to the low product inhibition, high activity in non-aqueous media, low reaction time, resistance to altered temperature, pH, alcohol, reusability of immobilized enzyme and being active without the aid of cofactors (Verma and Sharma, 2014).

2.2 Sources of lipase

Lipases are omnipresent enzymes which are found in animals, plants, fungi and bacteria. In the survey about the contribution of lipases in the industrial biotechnological applications showed that the contribution of bacterial lipases is 45%, fungal 21%, animal 18%, plants 11% and algae 3% (Patil *et al*., 2011). Animal lipases are rarely studied, but still they have been isolated from many insects (Horne *et al*., 2009), fishes (Görgün and Akpınar, 2012) and mammals (Vakhlu and Kour 2006). Animal lipases play an important role in the digestion of lipids in biological systems (Patil *et al*., 2012). Plant lipases mostly are present in the food reserve tissues of growing seedlings or especially in those which contains large amount of triacylglycerols (Pahoga and Sethar, 2002). Lipase activity in plant seeds increases during germination because the triacylglycerols are converted to soluble sugars by the action of lipase which is then transported to the growing tissues to supply structural carbon and energy to provide support for the growth of young plants (Patil *et al*., 2012).

Microorganisms producing lipases have been found in diverse habitats such as industrial wastes, vegetable oil processing factories, dairies, soil

contaminated with oil, oilseeds, and decaying food, compost heaps, coal tips, and hot springs (Wang *et al*., 1995). Microbial lipases are often more useful than enzymes derived from plants or animals because of the great variety of catalytic activities available, the high yields possible, are also more stable than their corresponding plant and animal enzymes and their production is more convenient and safer (Momsia *et al*., 2013). Most commercially important lipase-producing fungi are recognized as belonging to the genera *Rhizopus* sp., *Aspergillus* sp., *Penicillium* sp., *Geotrichum* sp., *Mucor* sp., and *Rhizomucor* sp. Lipase production by fungi varies according to the strain, the composition of the growth medium, cultivation conditions, pH, temperature, the kind of carbon and nitrogen sources used (Thakur, 2012). The extracellular bacterial lipases are of considerable commercial importance, as their bulk production is much easier and high yields (Gupta *et al*., 2004). Bacterial lipases are glycoprotein's but some extracellular bacterial lipases are lipoproteins, and most bacteria lipases are non-specific in substrate specificity and few are thermostable (Ghosh *et al*., 1996). Although a number of lipase-producing bacterial sources are available, only a few are commercially exploited as wild or recombinant strains. Of these, the important ones are: *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Chromobacterium* and *Pseudomonas*. Of these, the lipases from *Pseudomonas* bacteria are widely used for a variety of biotechnological applications (Gupta *et al*., 2004).

2.3 Industrial applications of lipases

The commercial production of enzymes from microbial sources and their environment friendly characteristics has convinced the industrial bodies to believe that enzymes are indeed a sustainable alternative to chemicals in industrial processes (Momsia *et al*., 2013). Microbial lipases proved to be biotechnologically valuable enzymes as they are versatile in their applied properties, ease of mass production, act under mild conditions, stable in organic solvents, broad substrate specificity, regio- and/or stereoselectivity, high stability compared to animal or plant lipases, higher enzymatic activity compared to fungi, tend to have optimal pH at neutrality or alkalinity and are mostly extracellular which facilitate their extraction from the fermentation medium (Bueno *et al*., 2014; Verma and Sharma, 2014; Momsia *et al*., 2013; Freitas *et al*., 2007). Lipases active under ambient conditions and the energy expenditure required to conduct reactions at elevated temperatures and pressures is eliminated that reduces the destruction of labile reactants and products, all these reasons make microbial lipase very attractive for industrial applications (Hasan *et al*., 2006).

Lipases are important enzymes used in many industrial applications such as pharmaceuticals, food processing, detergents formulations, paper and pulp, agrochemicals, bioremediation and biosurfactants (Sharma *et al*., 2011; Vakhlu and Kour , 2006) , development of fragrances, the manufacture of host skin-care products, and in most cosmetic products (Shakilabegam *et al*., 2012). In the food industry**,** lipases have been used for the flavour development in dairy products and flavour enhancement of cheeses (Pandey *et al*., 1999), production of cocoa butter equivalent, human milk fat substitute (Memosia *et al*., 2013), the acceleration of cheese ripening, the manufacturing of cheese like products, and the lipolysis of butterfat and cream (Shakilabegam *et al*., 2012). In addition, lipases utilized in the processing of other foods, such as meat, vegetables, fruit, baked foods, milk product and beer (Ray, 2012). In tea processing enzymatic breakdown of membrane lipids initiate the formation of volatile products with characteristic flavor properties (Verma and Sharma, 2014).

In oleochemical industry, lipases have been applied in many successful fat splitting/modification for the production of enormous oleochemicals. In these applications, usually the immobilized enzymes used to initiate various reactions (hydrolysis, alcoholysis, and glycerolysis) using mixed substrates. Using immobilized lipases saves energy and minimizes thermal degradation during hydrolysis, glycerolysis, and alcoholysis (Verma and Sharma, 2014; Hasan *et al*., 2006). In detergent industry lipases used as additives in laundry and household detergents (Sharma *et al*., 2014; Verma and Sharma, 2014; Hasan *et al*., 2006 ; Sharma *et al*., 2001). In pulp and paper industry lipases are used to remove the hydrophobic components of wood from the pulp produced for paper making (Sharma *et al*., 2001). In textile industry, lipases are used to assist in the removal of size lubricants, in order to provide a fabric with greater absorbency for improved levelness in dyeing. Its use also reduces the frequency of streaks and cracks in the denim abrasion systems (Hasan *et al*., 2006). In organic synthesis lipases used to catalyze a wide variety of chemo-, regio-, and stereoselective transformations for the production of fine chemicals (Sharma *et al*., 2001). Lipases as biosensors enable physicians precisely to diagnose patients with cardiovascular complaints (Verma and Sharma, 2014).

2.4 Biodiesel.

Biodiesel is defined as a fuel comprised of mono-alkyl esters of long-chain fatty acids derived from vegetable oils or animal fats (Adamczak *et al*., 2009). This biofuel offers several interesting and attractive properties, biodegradability and non-toxicity, compared to petroleum-based diesel. The

most important advantage of biodiesel, as a renewable material, is in maintaining a balanced carbon dioxide cycle. Additionally, biodiesel combustion results in reduced emission of carbon monoxide, sulfur, aromatic hydrocarbons and soot particles. On the other hand, several disadvantages for biodiesl were also reported for example the high viscosity, lower energy content, high cloud and pour point, high nitrogen oxide emission, lower engine speed and power, injector cooking, high price and engine erosion (Adamczak *et al*., 2009).

Biodiesel production is one of the stunning biotechnological applications of lipases. Lipase-catalyzed transesterification takes place in two steps, which involves hydrolysis of the ester bond and esterification with the second substrate. Lipase catalyzes the enzymatic production of biodiesel production through transesterification reaction in Figurer 1.2. In this reaction, an alcohol (usually methanol or ethanol) is added to the oil or fat in the presence of lipase catalyst, a mixture of glycerin and alkyl esters of fatty acids is generated, which is called biodiesel (Ribeiro *et al*., 2011). Although lipases from different sources are able to catalyze the transeterification reaction, bacterial and fungal lipases are mostly used in biodiesel production such as *Aspergillus niger*, *Candida antarctica*, *Candida rugosa*, *Chromobacterium viscosum*, *Mucor miehei*, *Pseudomonas cepacia*, *Pseudomonas fluorescens*, *Photobacterium lipolyticum*, *Rhizopus oryzae*, *Streptomyces sp.*, and *Thermomyces lanuginose* (Yahya*et al*., 1998).

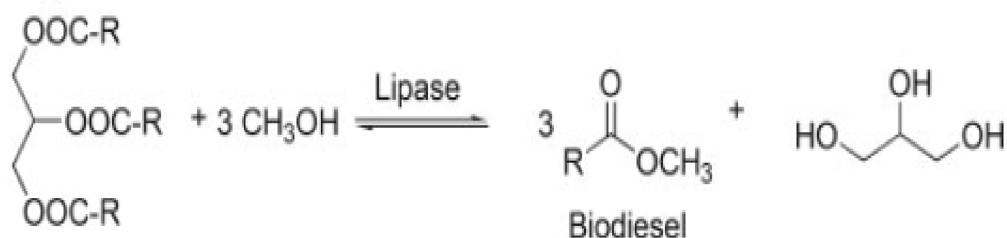


Figure 2.1 : Synthesis of biodiesel (fatty acid esters) by transesterification of a triglyceride (Adamczak *et al*., 2009).

2.5 Growth requirements for optimum lipase production .

Many studies have been undertaken to define the optimal culture and nutritional requirements for lipase production in submerged cultures. Bacterial lipases are mostly extracellular and are greatly influenced by various nutritional and physico-chemical factors, such as temperature, pH, nitrogen and carbon sources, presence of lipids, inorganic salts, agitation, and dissolved oxygen concentration (Gupta *et al*., 2004). The major factor for the

expression of lipase activity has always been carbon source , since lipases are inducible enzymes and are thus generally produced in the presence of a lipid source such as oil or any other inducers, such as triacylglycerols, fatty acids, hydrolyzable esters, tweens, bile salts and glycerol. It was observed that little enzyme activity was observed in the absence of olive oil even after prolonged cultivation of *Bacillus* sp. (Sugihara *et al*., 1991). On the other hand, good lipase yield was also reported in the absence of fats and oils in the fermentation media (Boonchaidung and Papone, 2013). In addition to the lipid inducers, bacterial lipases production is significantly influenced by other carbon sources, such as sugars, sugar alcohol, polysaccharides, whey, casamino acids and other complex sources (Rashid *et al*., 2001; Ghanem *et al*., 2000 ; Lotraku land Dharmsthiti, 1997; Gilbert *et al*., 1991).

Nitrogen sources and micronutrients should also be carefully considered for growth and enzyme production optimization. (Kumar *et al*. 2012) reported that peptone and yeast extract as nitrogen sources in the growth media supported the highest lipase production by various *Bacillus* sp. In another study, the results showed that yeast extract produced maximum enzyme activity, from *Penicillium citrinum*, followed by peptone, tryptone and beef extract (Hasan *et al*., 2001). The effect of nitrogen source on lipase production by *Bacillus licheniformis* indicated that the yeast extract was suitable substrate for accelerating lipase production (Larbidaouadi *et al*., 2014).

The effect of metal ions on lipase production by various microorganisms was studied by many researchers. The results showed that the requirement for metal ions vary with the organism. For instance, lipase production was stimulated from *Burkholderia sp*. in the presence of Ca^{2+} and Mg^{2+}. Also in *Bacillus sp.* RSJ1 the enzyme production was also stimulated in the presence of calcium chloride while, most other metal ion salts were inhibitory to lipase production (Sharma *et al*., 2002). Iron was found to play a critical role in the production of lipase by *Pseudomonas* sp. G6 (Kanwar *et al*., 2002). Again, the presence of Ca^{2+} alone or in combination with other ions such as Mg^{2+} and Fe^{2+}enhanced lipase production by a strain of *Bacillus* (Dhiman *et al*., 2013).

In addition to the various chemical constituent of the production medium, physiological parameters such as pH, temperature, agitation, and incubation period also play an important role in influencing lipase production by different microorganism. Lipase enzyme production was reported throughout bacterial growth with peak production being obtained by the late log phase. The production period for lipases varies from a few hours to a few days (Gupta *et al*., 2004; Sharma *et al*., 2001). It hasbeen reported that an

incubation period of 12 h was optimum for lipase production by *A. calcoaceticus* and *Bacillus* sp. RSJ1 and 16 h for *B. thermocatenulatus* while maximum lipase was produced after 72 h and 96 h of incubation, respectively, in the case of the *Pseudomonas fragi* and *P. fluorescens* BW96CC (Sharma *et al*., 2002; Mahler *et al*., 2000; Dong *et al*., 1999; Schmidt-Dannert *et al*., 1997; Pabai *et al*., 1996). (Kumar *et al*. 2012) reported maximum lipase production by *Bacillus* sp. MPTK 912 isolated from oil mill effluent in 72 h of incubation. (Larbidaouadi *et al*. 2014) reported that lipase production was the highest at incubation time of 48 hours by the lipase producing bacteria *Bacillus licheniformis*. In another study, maximum production of organic solvent-tolerant lipase from *Pseudomonas* sp. strain S5 was generated in the late logarithmic phase (after 20 h), and increased until optimum production was achieved, after 48 h of incubation (Rahman *et al*., 2006).

It was reported in the literature that the initial pH of the growth medium is one of the most important factors that influence lipase production. In (Jianrong *et a* 2011). stated that bacteria prefer pH around 7.0 for best growth and lipase production. However, maximum lipase production by several bacterial species at alkaline and acidic environments was also reported (Bora and Bora, 2012; Ray, 2012; Ramani *et al*., 2010;Sharma *et al*., 2001). From the point of temperature, it has been observed that, in general, lipases are produced in the temperature range of 20-45°C (Gupta *et al*., 2004). Other reports were showed that some other bacterial strains were also reported to produce maximum lipase enzyme outside this range (Joseph *et al*., 2006; Sharma *et al*., 2001).

The effect of agitation speed on lipase production was largely studied and most of the results concluded that the enzyme production greatly enhanced under shaking condition compared to the stationary conditions (Joseph *et al*., 2006; Bapiraju *et al*., 2005).This enhancement which increased with the increase in the agitation speed was related to the higher availability of the carbon source, enhanced aeration besides providing the nutrients required for the microbial cells to grow (Qureshi and Aslam, 2013; Joseph *et al*., 2006; Bapiraju *et al*., 2005). Regarding the inoculums size, it has been reported that the effect of inoculums size on lipase enzyme production is correlated with the total dissolved oxygen in the medium. Therefore, lipase production enhanced at low inoculum size due to the availability of sufficient nutrient and oxygen. Meanwhile higher inoculum size will cause insufficiency of total dissolved oxygen and nutrient supply which is subsequently causes decrease in lipase production (Baharum *et al*., 2003).

Chapter Three
Design and Methodology

3.1 Soil samples collection.

Total of 13 soil samples were collected from 7 different petrol stations and car repair shops in Al-karark province, south of Jordan. Roughly, 5 to 7 grams surface soil samples were collected in small sterilized plastic containers. The locations of soil sampling, contaminated with hydrocarbons residues, were selected based on the fact that microorganisms living in such environment potentially might produce lipase enzyme with organic solvent tolerance characteristics. However, the upper surface of the soil was removed and soil samples were collected from 2-3 cm beneath. The soil was brought to the laboratory and processed immediately.

3.2 Screening and selection of extracellular lipase producing bacteria.

The collected soil samples were screened for bacterial isolates with lipolytic activity using nutrient agar (NA) plates supplemented with 2% (w/v) tributyrin. Nutrient agar-tributyrin plates were prepared by dissolving the NA powder in distilled water and then the hot agar solution was homogenized with 2% (w/v) tributyrin using blender to create stable emulsion. The prepared emulsion was then autoclaved for 15 min at 121 ^{o}C and 15 psi.

For the purpose of isolation of bacterial isolates with lipolytic activity a serial dilution of the soil samples was performed as following. Soil samples were suspended in sterile distilled water by adding one gram of the soil sample to 10 mL of sterilized distilled water to form 1:10 (w/v) suspension followed by vigorous mixing for 5 min. Serial dilutions of 10^{-1} to 10^{-7} were prepared and 100 μL of 10^{-4} and 10^{-5} dilutions were spread on the surface of the nutrient agar-tributyrin plates.

The plates were incubated at 30°C and observed for 72 hours. Bacterial colonies with clear zones surrounding them were selected and transferred (streaking) to new NA-tributyrin plates. The plates were incubated at 30^{o}C for further purification of the selected bacteria. After isolation, the purified bacterial isolates were transferred to 20% (w/v) glycerol solution and stored at 4^{o}C till use.

3.3 Selection of methanol and ethanol tolerant lipase enzyme

3.3.1 Culture medium and growth conditions

Total of 34 bacterial isolates with lipolytic activity were obtained from the previous step. For the conformation of the extracellular lipolytic activity of

the selected isolates and as a source of lipase enzyme, the bacterial isolates were grown on slightly modified ($MgSO_4.7H_2O$ salt was replaced by Na_2SO_4) M9 minimal medium (Atlas, 2004) supplemented with 1.0% (w/v) olive oil as lipase inducer. The chemical composition and preparation of the olive oil-M9 medium was as following (per 100 ml): 0.6 g Na_2HPO_4, 0.3 g KH_2PO_4, 0.1 g NH_4Cl, 0.2 g glucose, 0.05 g NaCl, 0.014 g Na_2SO_4, 0.0014 g $CaCl_2$, 0.1 ml thiamine . HCl of 1.0 mg/mL solution and 1.0 g olive oil.

All the components above, except thiamine . HCl solution and olive oil, were dissolved in distilled water and mix thoroughly. pH was adjusted to 7.0. Olive oil 1.0% (w/v) was weighed first in the flasks and then the requested volumes of the mixture of the remaining components was added to each flask and then the mixture was autoclaved for 15 min at 15 psi pressure and121°C. After that the sterilized medium was cooled to room temperature and aseptically 1 ml volume of filter sterilized thiamine .HCl solution (1 mg/mL) was added to each flask. Loop-full of the selected bacterial isolates with lipolytic activity from pure cultures were inoculated into 100 ml olive oil-M9 medium (pH 7.0) and incubated in orbital shaker operated at 30 ^{o}C and 150 rpm rotation speed for 72 hours. Five ml sample have been withdrawn from the culture medium every 24 hours and lipase activity was measured using the assay method described by (Winkler and Stuckmann, 1979).

3.3.2 Enzyme immobilization

Immobilization of the lipase enzyme from the supernatant of the 34 cultured bacterial isolates was performed on cellulose powder. The immobilization step was performed for the purpose of concentrating the enzyme and to be used in the following experiments for the selection of methanol and ethanol tolerant lipase producing bacteria. The supernatant from the withdrawn samples with the highest lipase activity (24, 48 and 72 hour's samples withdrawn during the time profile described in the previous section) were used in the immobilization experiment. The immobilization was performed by wetting the cellulose powder with the lipase crude supernatant in 1:4 (w:v) ratios, respectively. The wetted cellulose powder was left to air dry at room temperature for 48 hours. Ten milligram of the immobilized enzyme powder was used to measure the lipolytic activity of the immobilized enzyme powder using the lipase assay method described by Winkler and Stuckmann (1979). Before the measurement of the color intensity of the assay solution at 410 nm, the assay solution was centrifuged at 10,000 rpm for 1 min to precipitate the cellulose powder.

3.3.3 Methanol and ethanol stability test.

For the selection of methanol and ethanol tolerant lipase enzyme, the immobilized lipase powder was used in this experiment. The selection of methanol and ethanol tolerant lipase enzyme was performed in two steps experiment. In the first step, 100 mg of the immobilized enzyme powder was covered with considerable amount of methanol and ethanol separately and left at 30 ^{o}C for 24 hours for the organic solvents to evaporate. After that, 10 mg of the treated enzyme powder was used in the lipase assay experiment to determine the residual activity of the lipase enzyme after exposure to methanol and ethanol hydrophilic organic solvents.

In the second step, 25, 50 and 75% (v/v) concentrations of the tested hydrophilic organic solvents, methanol and ethanol, was added separately to the assay mixture to determine the relative activity of the lipase enzyme in presence of different concentrations of methanol and ethanol comparing to the control experiment. The control experiment was the assay mixture with the immobilized enzyme powder without adding methanol and ethanol.

3.4 Identification of methanol and ethanol tolerant lipase producing bacteria.

For the purpose of identification of the bacterial isolate K5b4, which selected based on the lipase stability test in presence of methanol and ethanol organic solvents, microscopic studies, Gram stain as well as oxidase enzyme test were performed. Based on the results of the previous tests RapID™ ONE System (Thermo Fisher Scientific, USA) was used to identify the selected bacterial strain to the genus and species level. In addition, the identification of the bacterial strain K5b4 was confirmed using the 16S rRNA gene sequencing (GENEWIZ , USA).

3.5 Optimization of the physical and nutritional parameters affects extracellular lipase production from *Acinetobacter* sp. K5b4.

3.5.1 Effect of inoculum size on extracellular lipase production.

To evaluate the effect of the inoculum size on lipase enzyme production by the bacterial isolate K5B4, different (v/v %) inoculums sizes was studied. To prepare the seed culture as the source of the inocula, the M9-olive oil medium (pH 7.0) was inoculated with loopful of the k5b4 bacteria and incubated at 30^{o}C and 150 rpm for overnight. The following 0.2, 0.4, 0.6 and 0.8% (v/v) inocula from 0.2 OD_{600} seed culture were inoculated into 100 ml fresh M9-olive oil culture medium prepared in 250 ml Erlenmeyer flasks. The inoculated culture medium (pH 7.0) was then incubated at 30^{o}C and 150 rpm for 48 hours. Lipase enzyme production was determined using the

colorimetric assay method developed by (Winkler and Stuckmann, 1979) and described in section 3.6.1. The bacterial growth as dry weight biomass (g/L) was measured as described in section 3.6.2.

3.5.2 Effect of initial pH on extracellular lipase production.

To investigate the effect of initial pH of the culture medium on the growth and lipase production by isolate K5B4, the pH of the culture medium was adjusted to 5.5, 6.0, 6.5, 7.0 and 7.5 using 2.0 N NaOH and HCl. 100 ml of the growth medium, M9-olive oil, was inoculated with 0.4% (v/v) inocula from 0.2 OD_{600} seed culture and then incubated in orbital shaker operated at 30 ^{o}C and 150 rpm for 48 hours. Lipase production and bacterial growth was measured as described in section 3.6.1 and 3.6.2, respectively.

3.5.3 Effect of agitation speed on extracellular lipase production.

The effect of agitation speed on the growth and lipase production by isolate k5b4 was studied by varying the rotation speed of the shaker incubator by 50, 100, 150 and 200 rpm. The M9-olive oil culture medium (pH 7.0) was inoculated with 0.4 % (v/v) seed culture (0.2 OD_{600}) and incubated in orbital shaker operated at 30 ^{o}C and different rotation speeds for 48 hours. Lipase production and bacterial growth was measured as described in section 3.6.1 and 3.6.2, respectively.

3.5.4 Effect of incubation temperature on extracellular lipase production.

The effect of incubation temperature on lipase enzyme and biomass production by isolate k5b4 was evaluated by incubating the bacterial culture in orbital shaker operated at different temperatures (27, 30, 33 and 35 ^{o}C). The M9-olive oil culture medium (pH 7.0) was inoculated with 0.4% (v/v) seed culture and incubated in shaker incubator operated at 150 rpm and different operating temperatures for 48 hours.

3.5.5 Effect of different lipase inducers on extracellular lipase production.

For the evaluation of the effect of lipase inducers on lipase production by the bacterial isolate k5b4, different natural and synthetic lipid inducers (1.0% w/v) were supplemented to the growth medium to investigate their induction ability. Olive oil and corn oil purchased from the local market have been used as natural inducers in this experiment. Meanwhile tributyrine, Tween 20 and Tween 80 were used as synthetic inducers. Due to their probable toxic effects at high concentrations, tributyrin, Tween 20 and Tween

80 were supplemented to the growth medium with different concentrations of 0.25, 0.5, 0.75 and 1.0% (w/v). The effect of different concentrations of the best inducer obtained from the previous experiment was further studied by supplementing the growth media with 0.25, 0.5, 0.75, 1.0 and 1.5% (w/v) concentrations.

3.5.6 Effect of glucose concentration on extracellular lipase production.

Due to the facts that (1) Glucose as a carbon source in the growth media can cause catabolic repression and represses the production of many metabolites (2)Even though it shows the optimum operating parameters, changing the growth conditions in the previous experiments did not show any enhancement in the level of lipase enzyme production. In this experiment the investigation of the existence of catabolic repression on lipase production due to the presence of glucose was studied. Different concentrations (0.05, 0.1, 0.15 and 0.2% w/v) of glucose were added to the culture media and the production of the lipase enzyme was compared to the control experiment. The control experiment was the M9-oilive oil media without glucose. The growth media (pH 7.0) was inoculated with 0.4 % (v/v) seed culture (0.2 OD_{600}) and incubated at 30 ^{o}C and 150 rpm for 48 hours in orbital shaker.

3.5.7 Effect of different carbon sources on lipase production.

In order to study the effect of carbon sources (other than lipids) on the enzyme and biomass production, the following carbon sources starch, glycerol, sorbitol, mannitol, maltose, fructose, lactose and sucrose were added (0.2% w/v) to the culture media that contain olive oil as the sole carbon source. The growth media (pH 7.0) was inoculated with 0.4% (v/v) seed culture (0.2 OD_{600}) and incubated at 30 ^{o}C and 150 rpm for 48 hours in orbital shaker. In subsequent experiment different concentrations (0.1, 0.2, 0.3 and 0.4% w/v) of the best carbon sources that supported the highest lipase enzyme production were studied. The enzyme production was compared to the control experiment which was the M9-olive oil media without carbon source.

3.5.8 Effect of different nitrogen sources on lipase production.

In this experiment, the effect of 0.1% (w/v) of different nitrogen sources (NH_4Cl, peptone, tryptone, urea, yeast extract, ammonium nitrate and $NaNO_3$) on the enzyme and biomass production was studied. The growth media (pH 7.0) composed of (per 100 ml): 0.6 g Na_2HPO_4, 0.3 g KH_2PO_4, 0.2 g glycerol, 0.05 g NaCl, 0.014 g Na_2SO_4, 0.0014 g $CaCl_2$, 0.1 ml thiamine.HCl of 1.0 mg/ml solution and 0.1 g of different nitrogen sources. The growth media was inoculated with 0.4% (v/v) seed culture (0.2 OD_{600})

and incubated at 30 ^{o}C and 150 rpm for 48 hours in orbital shaker. In another experiment, the effect of different concentrations (0.05, 0.1, 0.15, 0.2, 0.25 and 0.3% w/v) of the best nitrogen source on lipase production was studied. The enzyme production in the test experiment was compared to the control experiment which was the growth media without nitrogen source.

3.5.9 Effect of mineral salts and NaCl concentration on lipase production.

In this experiment, a study was carried out into the effects of five mineral elements (Mg^{2+}, Ca^{2+}, K^{+}, and Mn^{2+} and Co^{2+}) on the lipase enzyme and biomass production by the bacterial isolate k5b4. The mineral elements were added separately to the growth media. With the aim of establishing the influence of NaCl concentration on the lipase enzyme and biomass production by the bacterial isolate K5b4, different concentrations (0.025, 0.05, 0.75, 0.1, 0.2, 0.3, 0.4 and 0.5% w/v) of NaCl were added to the growth media. In both experiment the growth media was inoculated with 0.4% (v/v) seed culture (0.2 OD_{600}) and incubated at 30 ^{o}C and 150 rpm for 48 hours in orbital shaker.

3.6 Analysis.

3.6.1 Enzyme assay.

Lipase activity was measured spectrophotometrically as described by Winkler and Stuckmann (1979) with some modifications. 0.5 mM of *p*-nitrophenyl laurate were prepared in 10 mL of dimethyl sulfooxide (DMSO) and emulsified in 90 mL of 100 mM phosphate buffer (pH 7.0), 0.1% (w/v) polyvinyl alcohol (PVA) and 0.4% (w/v) Triton X-100. One mL of the crude enzyme was added to 2.0 mL of the reaction mixture and then incubated for 10 min at 30 ^{o}C. The color change was measured at 410 nm and compared with the *p*-nitrophenol standard curve. One unit (U) of lipase activity was defined as the amount of the enzyme that released 1 μmol of *p*nitrophenol per min.The *p*-nitrophenol standard curve was constructed by plotting the *p*-nitrophenol concentration (nmol) versus the color intensity at 410 nm. Different concentrations of *p*-nitrophenol (100, 200, 300, 400, 500, 600, 700 and 800 nmol) were prepared (triplicate) in 100 mM phosphate buffer (pH 7.0). The intensity of the color developed was measured at 410 nm using spectrophotometer.

3.6.2 Estimation of growth (dry weight biomass).

The dry weight biomass production in the growth culture of the bacterial isolate k5b4 was determined by measuring the OD of the culture broth at 600 nm and the readings were compared to the dry weight biomass

standard curve. The dry weight biomass standard curve was constructed by plotting the dry weight biomass versus bacterial culture OD_{600}. The OD_{600} of the k5b4 bacterial growth in M9-olive oil media was adjusted to 0.1, 0.2, 0.3, 0.4 and 0.5 by adding fresh media to the culture until these values were obtained. 50 ml of the culture media with the adjusted OD_{600} was centrifuged in pre-weighed dry polyethylene centrifuge tubes at 5000 rpm for 10 min. The supernatant was discarded and the biomass pellet was dried in incubator operated at 35 ^{o}C. The weight of the pellet was measured every 24 hours until stable reading was obtained. The measurement of the dry weight biomass was performed in duplicate.

Chapter Four
Findings and Discussion and Recommendations

4.1 Screening and selection of extracellular lipase producing bacteria

In this work 13 soil samples were collected from different petrol stations and car repair shops in Al-karark province, south of Jordan. The collected soil samples were screened for extracellular lipase producing bacteria using nutrient agar supplemented with 2% (w/v) tributyrine. The outcome of this screening was 34 bacterial isolates with the extracellular lipolytic activity.

The extracellular lipolytic activity of these bacterial isolates was identified through the clear hydrolytic zone surrounding the bacterial colonies on NA-tributyrine plates (Figure 4.1, 4.2, 4.3, 4.4, 4.5, 4.6 and 4.7). Thus the appearance of clear zones that surrounding the bacterial isolates were taken as a sign of the hydrolytic activity of the secreted extracellular lipase using the synthetic triacylglycerol (tributyrine) as substrate that was supplemented to the nutrient agar plates.

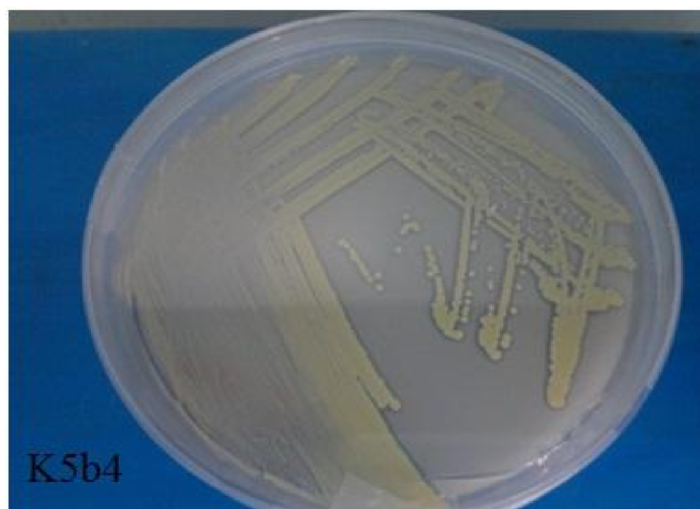


Figure 4.1: The hydrolysis zone around the bacterial colonies of K5b4 isolate

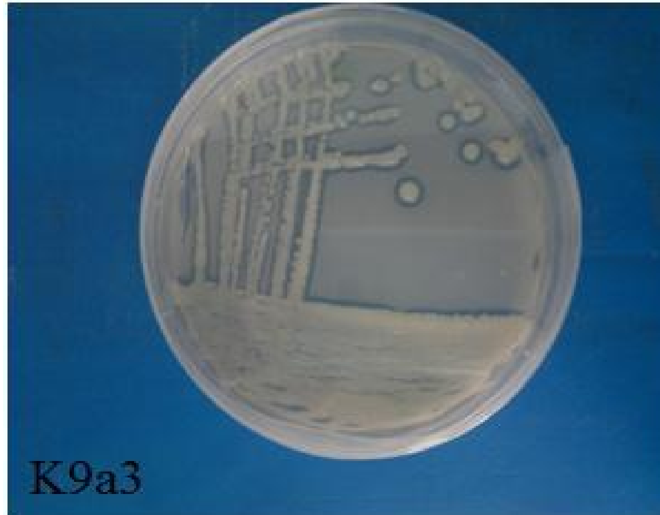


Figure 4.2: The hydrolysis zone around the bacterial colonies of K9a3 isolate

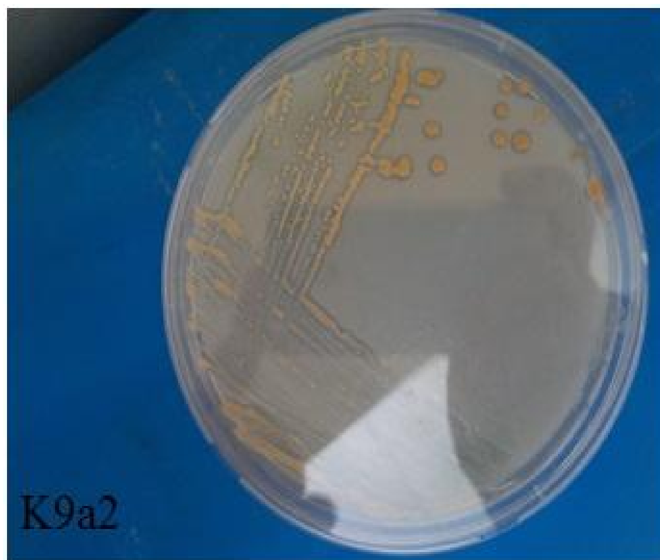


Figure 4.3: The hydrolysis zone around the bacterial colonies of K9a2 isolate

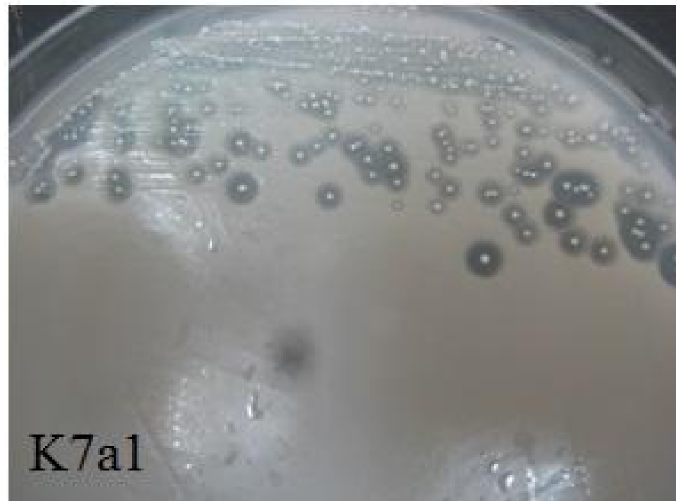


Figure 4.4: The hydrolysis zone around the bacterial colonies of K7a1isolate

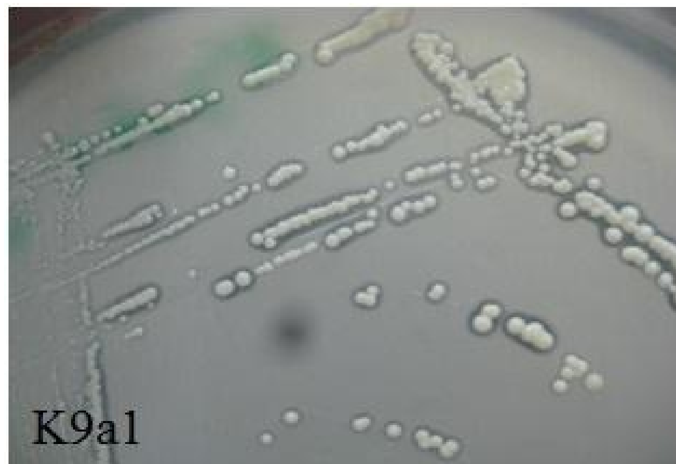


Figure 4.5: The hydrolysis zone around the bacterial colonies of K9a1 isolate

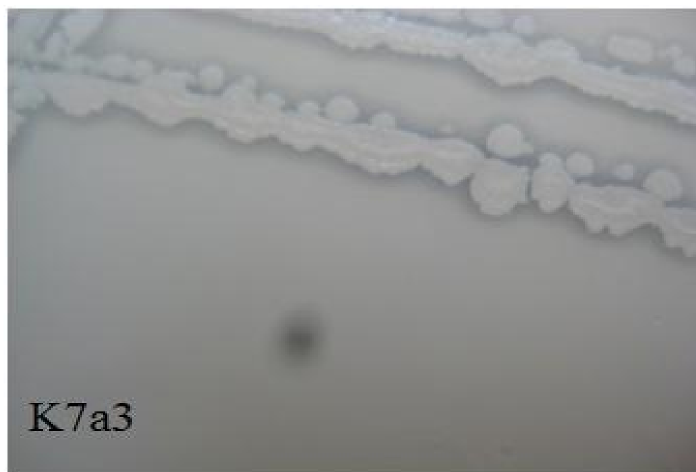


Figure 4.6: The hydrolysis zone around the bacterial colonies of K7a3 isolate

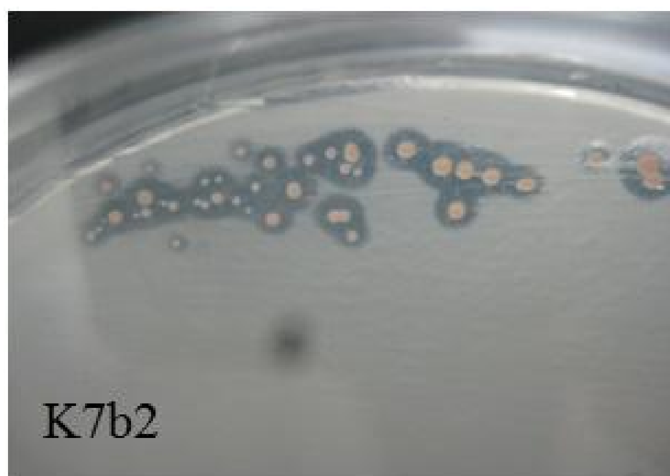


Figure 4.7: The hydrolysis zone around the bacterial colonies of K7b2 isolate

4.2 Selection of methanol and ethanol tolerant lipase-producing bacteria.

All the 34 isolated bacteria with extracellular lipolytic activity resulted from the screening step were grown on M9-olive oil media. Previous to the organic solvent stability test experiment, the crude lipase enzyme obtained through the centrifugation (5000 rpm for 10 min) of the culture media was immobilized on cellulose powder. The lipolytic activity of the immobilized enzyme was confirmed by the enzyme assay described by (Winkler and Stuckmann, 1979) using *p*-nitrophenyl laurate as substrate.

The immobilized enzyme from the 34 isolates was used in two step experiment for the selection of methanol and ethanol tolerant lipase producing bacteria. From the first step, the list of the selected bacterial isolates was shortened to seven bacterial isolates namely K9a3, K9a2, K7a1, K9a1, K7a3, K5b4 and K7b2. These seven isolates were selected based on the results of the stability experiment of their lipases after it have been exposed to the hydrophilic organic solvents, methanol and ethanol. The immobilized lipase preparations of these 7 isolates maintained 100% of their original activity as compared to the control experiment. However, the immobilized enzyme preparations of the remaining isolates were showed reduction in their residual activity in the range of 23 to 77% as compared to the control experiments (Data not shown).

In the second step, the immobilized lipases of the seven bacterial isolates selected from the first step were further subjected to the evaluation of their relative lipolytic activity in the presence of different volume/volume percentages of methanol and ethanol in the assay solution. The results Figure 4.8 showed that in the presence of 25, 50 and 75% (v/v) methanol the immobilized lipase preparations of the isolates K9a2, K7a1, K9a1, K7a3, K5b4 and K7b2 were not just able to maintain their original activity at all the concentrations tested but showed high activation degree that increased with the increase in methanol volume percentage. Among the 6 isolates the enzyme produced by the bacterial isolate K5b4 showed the highest degree of stability and activation. On the other hand, the presence of 25% (v/v) of methanol in the assay solution of the immobilized lipase of the bacterial isolate K9a3 caused reduction in the lipolytic activity by 40%. Meanwhile, the presence of 50% (v/v) methanol did not affect the enzyme activity. Slight activation of 10% in the enzyme activity was observed when 75% (v/v) methanol was present in the assay solution.

On the other hand, when 25% (v/v) ethanol was added to the assay solution, four immobilized enzyme preparations were able to maintain the original activity namely isolates K7a1, K9a1, K7a3 and K5b4. Among the four isolates, the immobilized enzyme of K7a1 and K7a3 showed certain degree of activation by 14 and 50%, respectively. On the other hand, the immobilized enzymes of the remaining three isolates (K9a3, K9a2 and K7b2) were showed reduction in their relative activity in the range of 15 to 45%. When 50% (v/v) ethanol was applied, all the immobilized enzyme preparations suffered from the reduction in their relative activity except the immobilized enzyme of the bacterial isolate K7a3 which was not only able to retain the original activity but also showed 32% enhancement in the lipolytc

activity. Slight increase in the lipolytic activity of the immobilized enzyme of the bacterial isolate K9a3 was observed when 50% (v/v) of ethanol was applied comparing to 25%. When75% (v/v) of ethanol was tested, all the immobilized enzyme preparation without exceptions showed reduction in the lipolytic activity with the highest stability showed by the immobilized enzyme preparation obtained from the bacterial isolate K5b4 which was able to retain almost 60% of its original activity. Based on the results obtained from the previous experiments, the bacterial isolate K5b4 was selected for further study.

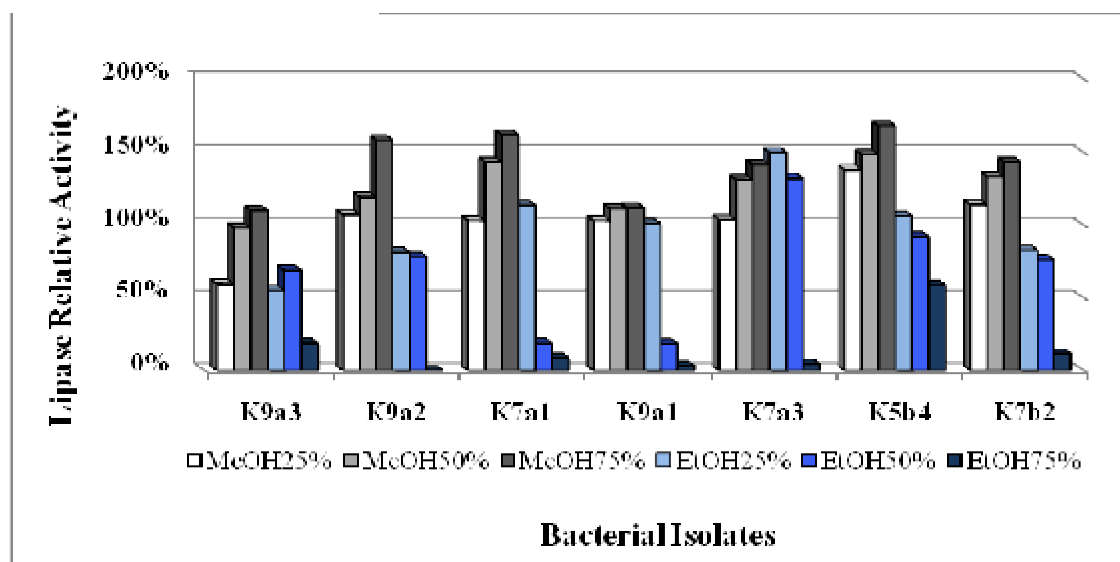


Figure 4.8: Immobilized lipase relative activity of the selected bacterial isolates in presence of different v/v percentage of methanol and ethanol in the assay solution. The control experiment was the assay experiment of the immobilized enzyme without the presence of methanol and ethanol.

Although it was previously reported that good stability of bacterial and fungal lipases in hydrophilic organic solvents is rare (Zhao *et al*., 2008). Our results showed that seven bacterial isolates were able to retain their original activity after exposure to the hydrophilic organic solvent methanol and ethanol. Furthermore, the presence of different concentrations of methanol and ethanol in the assay solutions of the selected seven bacterial isolates showed that the effect is varied between inhibitory, activation and no effect. However, it have been reported that the effect of the organic solvent on the lipase stability and activity depends on the nature of both enzyme and solvent (Hazarika *et al*., 2002) as well as the concentration applied (Baharum *et al*., 2006).

As one of the most important characteristics of lipases, the stability in organic solvents will determine whether the enzyme can be used to catalyze synthetic reactions and also which solvent would be better to perform the reaction (Lima *et al*., 2004). Hydrophilic organic solvents are usually incompatible with enzyme activity as water-miscible organic solvents strip off water molecules from the enzymes surface (Azevedo *et al*., 2001; Mozhaev *et al*., 1989). The strip off of water molecules lead to the unfolding of the enzyme molecule, denaturation, due to the exposure of the inner hydrophobic residues. this denaturation occurs at a much faster rate in organic solvents-aqueous system than in a pure aqueous system (Azevedo *et al*., 2001). This sudden drop in the enzyme activity at a critical concentration of organic co-solvent as a result of protein denaturation is a general phenomenon occurring in mixed aqueous solvents (Mozhaev *et al*., 1989).

Meanwhile the activation of lipases in the presence of organic solvents can be explained by the disruption of aggregates formed between the enzyme and lipids of the fermentation medium or between the enzyme molecules themselves (Sugihara *et al*., 1992) or due to the presence of residues of these solvents which can interact with hydrophobic amino acid residues present in the lid that covers the catalytic site of the enzyme, thereby maintaining the lipase in its open conformation (Rúa *et al*.,1993). (Gaur *et al*. 2008) reported that the stability/activation of lipases in polar solvents like methanol and ethanol has rarely been observed. The polar solvent tolerant lipases therefore appear promising for catalysis in low water medium and this property is observed as novel attribute of lipases which is present only in few cases.

However, the stability of few lipases from different bacterial species in presence of methanol and ethanol and other hydrophilic organic solvents was previously reported. It have been reported that the presence of 30% (v/v) of different organic solvents (methanol, acetonitrile, ethanol, acetone, 2-propanol, ethyl acetate and hexane) in the assay mixture of lipase enzyme produced by *Acinetobacter* sp. enhanced the lipase activity as follows: methanol (114.7%), acetonitrile (105.9%), ethanol (153.4%), acetone (133.9%), 2-propanol (135.9%), ethyl acetate (111.4%), and 114.0% with hexane (Khoramnia *et al*., 2011). (Schmidt-Dannert *et al*. 1997) were also reported activation in the lipolytic enzyme activity of *Bacillus thermocatenulatus* of 18% observed after addition of 30% methanol. In addition, the lipase from *Pseudomonas fluorescens* JCM5963 was activated in the presence of short carbon chain alcohols including methanol and ethanol (Zhang *et al*., 2009). In another report, the lipase of *Pseudomonas aeruginosa* was found to be stable in the presence of 25% of methanol and ethanol (Gaur *etal*., 2008). Also the lipase isolated from *Serratia marcescens* ECU1010

exhibited stability in the presence of 10% (v/v) methanol (Zhao *et al*., 2008). Finally, lipase produced by *Bacillus megaterium* showed activation in the *p*-nitrophenyl palmitate hydrolysis after incubation in increasing concentration of ethanol (Lima *et al*., 2004).

4.3 Identification of the selected bacterial isolate K5b4.

The selected bacterial isolate K5b4 was characterized as Gram negative, oxidase negative bacilli bacteria. However, the identification results obtained from the RapID™ ONE System (Kit) revealed that (Appendix I) the bacterial strain K5b4 is *Acinetobacter* sp. with more than 99.9 probability percentage as *Acinetobacter calcoaceticus*. Accordingly, the partial 16S rRNA sequence of the isolated strains was compared with those available in the Blast . Identification to the genus level was determined as a 16S rRNA sequence similarity of >97% *Acinetobacter* species.

4.4 Effect of inoculum size, initial pH, agitation speed and incubation temperature on lipase production from *Acinetobacter* sp. K5b4.

The effect of the inoculum size on lipase production by the bacterial isolate *Acinetobacter* sp. K5b4 was studied by inoculating the growth media with 0.2, 0.4, 0.6 and 0.8% (v/v) of 0.2 OD_{600} seed culture. The results showed in the Figure 4.9 indicated that the highest lipase production (2.8 mU/mL) was obtained when the culture medium was inoculated with 0.4% (v/v). Inoculum size below and above 0.4% (v/v) were clearly resulted in less enzyme production. On the other hand the growth of the bacteria was enhanced with the increase in the inoculum size with the highest biomass of 1.97 g/L dry weight was obtained when 0.8% (v/v) inocula was used.

According to (Baharum *et al*. 2003) inoculum size is correlated with the total dissolved oxygen in the medium. At low inoculum size, the nutrient and oxygen levels were sufficient for the growth of bacteria and therefore, enhanced lipase production. Meanwhile higher inoculum size will cause insufficiency of total dissolved oxygen and nutrient supply which is subsequently causes decrease in lipase production. In correlation, (Giuseppin 1984) had proved that the lipase production rate would be higher at low inoculums sizes and relatively high oxygen concentration. Similar pattern of results were reported by (Bisht *et al*. 2012) when they studied the effect of inoclulum size on extracellular alkaline lipase production by an improved strain of *Pseudomonas aeruginosa* MTCC 10,055 and they found that lipase production increased with an increase in inoculum size with the maximum lipase production (348.6 U/ mL) was obtained at 0.5% (v/v) inoculum size,

however, further increase in inoculums size resulted in sharp decrease in lipase production.

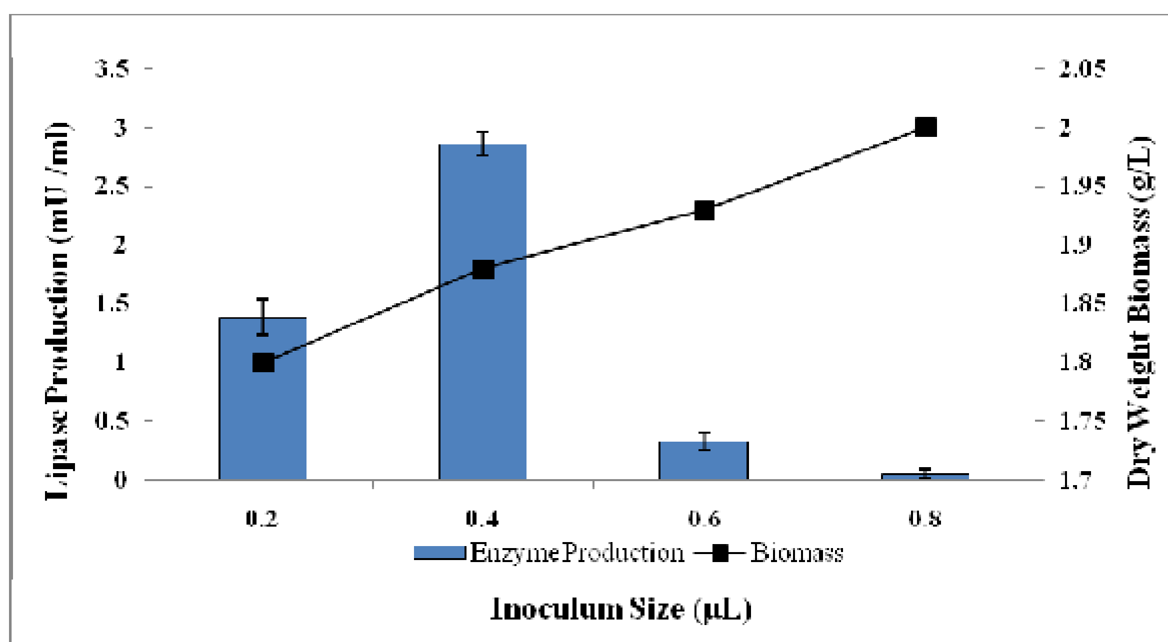


Figure 4.9: Effect of inoculum size on lipase production by*Acinetobacter* sp. K5b4**.** Bars presented for lipase production are mean values of ± standard deviation of triplicates.

Initial pH of the growth media considered as one of the critical factors that profoundly affect the growth and enzymes production. In this work the initial pH of the culture media of *Acinetobacter* sp. K5b4 was investigated for their effect on lipase production. As shown in (Figure 4.10), the results revealed that the highest enzyme production of 2.3 mU/ml was obtained when the bacterial culture grown on pH 7.0 with biomass of 1.79 g/L. Furthermore, the results showed that further increas in the intial pH of the culture media to 7.5 caused reduction in the enzyme and biomass production by 32.3 and 91%, respectivelly. On the other hand, when the initial pH of the culture media was adjusted to a value below 7.0, maximum biomass production of 3.41 g/L was recorded at initial pH of 6.5 which is not corrosponding with the enzyme production of 0.29 mU/ml that was much less than that obtained when the intitial pH of the culture media was 7.0. Similarly, the extracellular lipase enzyme production at initial pH of 6.0 and 5.5 was poorly detected in the fermentation media and also caused reduction in the biomass production.

Nevertheless, several reports showed that the degree of lipase production by *Acinetobacter* species as a function of initial pH is varied from

one species to another with neutral pH more common. For instance and similar to our results (Jagtap *et al*. 2010) were reported that maximum lipase production of 41.57 U/ml from *Acinetobacter haemolyticus* TA 106 was displayed at pH 7.0. In agreement with these results the maximum lipase production from *Acinetobacter* sp. in submerged fermentation process was also observed at pH 7.0 (Khoramnia *et al*., 2011). In addition, the maximum production of alkaline lipase produced by *Acinetobacter radioresistens* was also recorded at optimal pH of 7.0 (Chen *et al*., 1998). In contrast, (Anbu *et al*. 2011) reported that the two strains of *Acinetobacter* studied (BK44 and BK43) were prefer the production of lipase at acidic pH with the highest level of lipase production at pH 6.0.

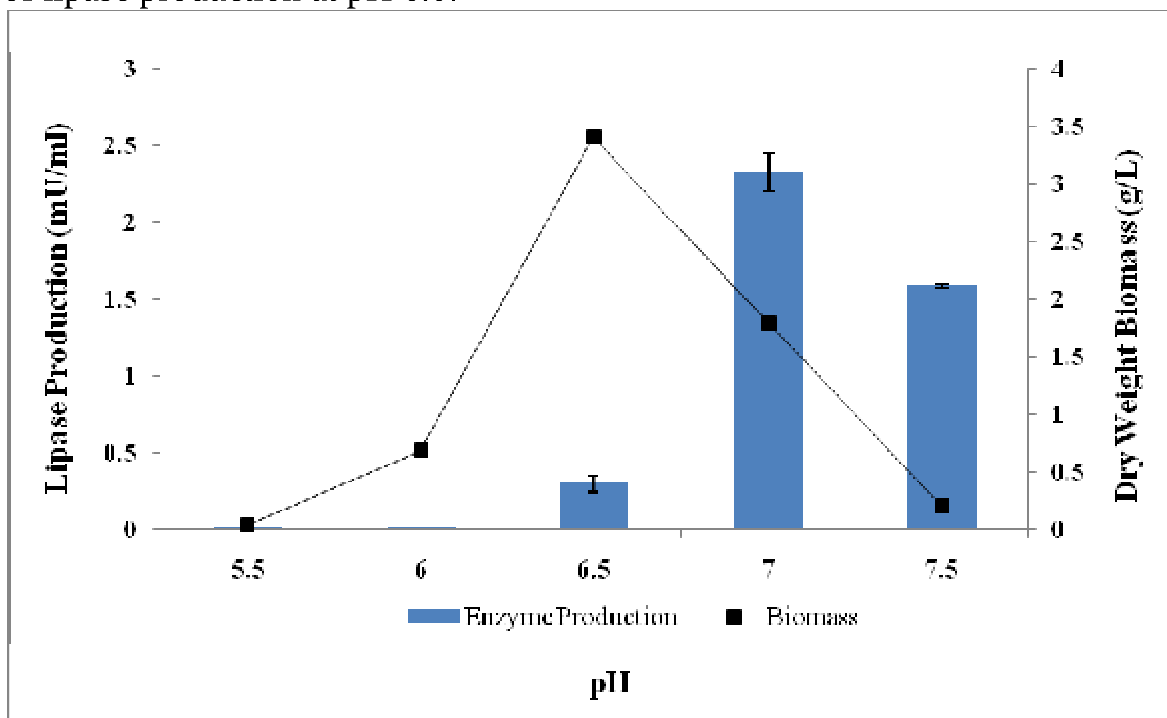


Figure 4.10 : Effect of initial pH of the culture media on lipase production by *Acinetobacter* sp. K5b4**.** Bars presented for lipase production are mean values of ± standard deviation of triplicates.

However, when the agitation speed in the running conditions of the bacterial culture was varied from 50 to 200 rpm, the results Figure 4.11 were clearly revealed that the highest extracellular lipase production was obtained when the bacterial culture was agitated under 150 rpm agitation speed with maximum enzyme production of 2.68 mU/ml and maximum biomass production of 2.0 g/L. With the compare with the optimum agitation speed, the results showed that running the culture at agitation speed above or below 150 rpm caused remarkable reduction in the enzyme production and slight

decrease in biomass production at 100 and 200 rpm. Meanwhile very sharp decrease in biomass and enzyme production was recorded when the rotation speed of the shaker incubator was set at 50 rpm. Previous studies showed that the significant increase in lipase production by the cultivated microorganism under shaking conditions compared to the static conditions may be due to the higher availability of the carbon source to the cells and further mixing of the medium enhanced aeration necessary for the microbial cells to grow (Qureshi and Aslam, 2013; Long *et al*., 2007; Joseph *et al*. 2006; Bapiraju *et al*. 2005; Elibol and Ozer, 2000).

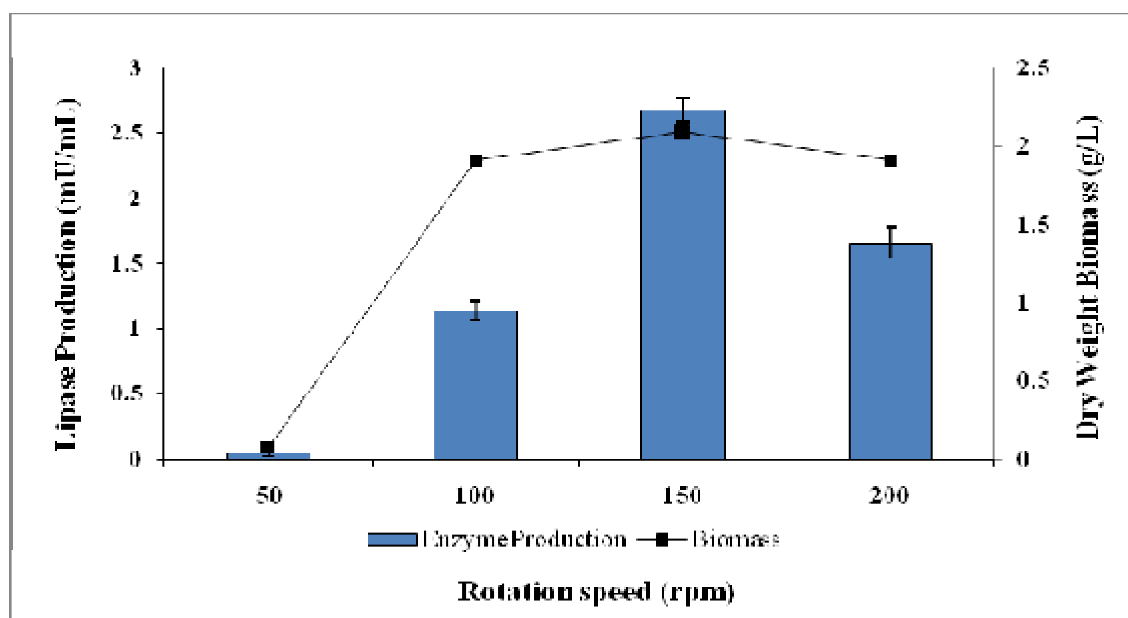


Figure 4.11: Effect of agitation speed of the culture media on lipase production by *Acinetobacter* sp. K5b4**.**

When the incubation temperature of the bacterial culture was varied, the results Figure 4.12 showed that, the incubation temperature of the growth media is critically important for the extracellular lipase production from *Acinetobacter* sp. K5b4. As it can be seen from the results, the temperatures below or above 30 ^{o}C were severely caused reduction in the enzyme production to levels were the enzyme activity were hardly can be detected in the culture supernatant. With un-corresponding relationship between the maximum enzyme production and maximum biomass yield, the highest enzyme production of 2.8 mU/ml was detected in the supernatant when the bacterial culture incubated at 30 ^{o}C, meanwhile the maximum biomass production of 2.4 g/L was obtained at 33 ^{o}C. Similar pattern of results were addressed previously (Figure 4.3) where the maximum production of enzyme

obtained at pH 7.0 meanwhile the maximum biomass production of biomass was at pH 6.5.

However, it has been reported that many species of *Acinetobacter* were psychrophilic and psychrotrophic (Park *et al*., 2009; Kasana *et al*., 2008). (Anbu *et al*. 2011) studied the effect of temperature on lipase production in two strains of *Acinetobacter* (BK43 and BK44) and found that the highest production by BK43 was obtained at 30°C, while the highest production by BK44 was obtained at 25°C. In the case of BK44, approximately 25% of the enzyme activity was lost when the temperature increased from 25 to 30°C. In addition, the enzyme production by both strains has decreased dramatically at 37°C, which indicates that these strains are unable to grow and produce lipase at higher temperatures. In another report, (Jagtap *et al*. 2010) documented a good lipase production by *Acinetobacter haemolyticus* isolated from healthy human skin was obtained at temperature of 28 to 40 ^{o}C with the highest value of lipase production recorded at 30 ^{o}C. In addition, the maximum production of a solvent, detergent, and thermo tolerant lipase by a newly isolated *Acinetobacter* sp. in submerged and solid-state fermentations was obtained at 29 and 30 ^{o}C, respectively (Khoramnia *et al*., 2011).

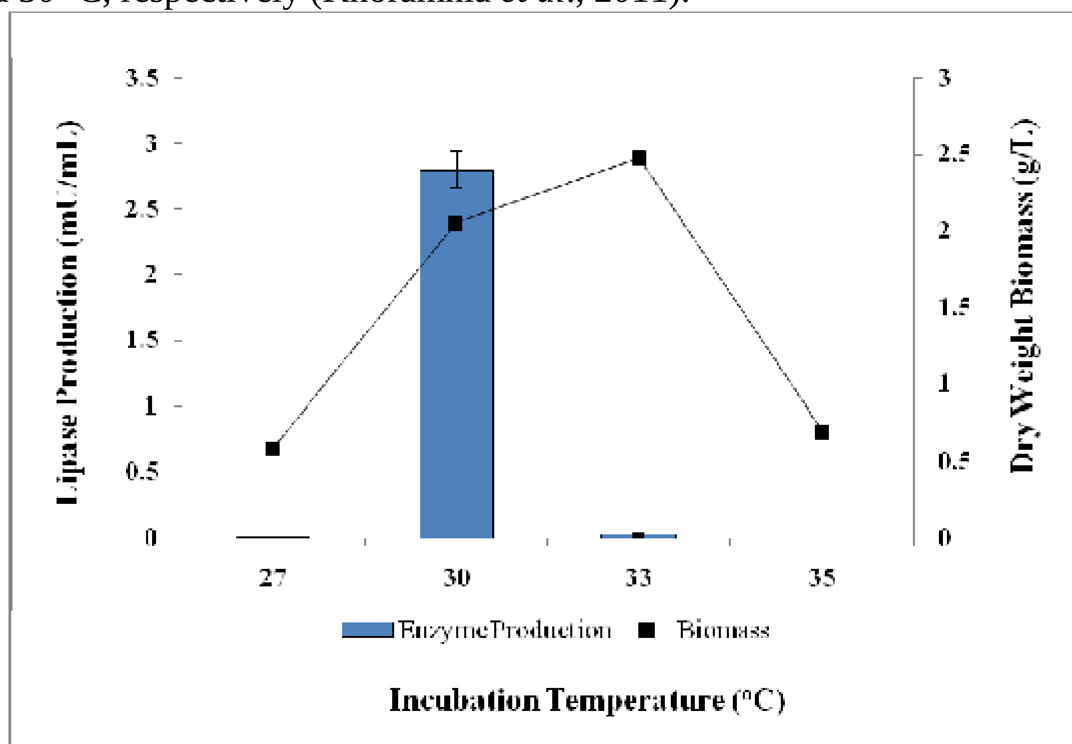


Figure 4.12: Effect of incubation temperature of the culture media on lipase production by *Acinetobacter* sp. K5b4**.** Bars presented for lipase production are mean values of ± standard deviation of triplicates.

4.5 Effect of inducers on lipase production from *Acinetobacter* sp. K5b4

Lipases are inducible enzymes and their production is largely depending on the presence of their inducers in the fermentation media. Several natural and synthetic lipase inducers have been reported in the literatures that induce the enzyme production in many folds. In this experiment different lipase inducers including natural and synthetic ones were supplemented to the fermentation media of *Acinetobacter* sp. K5b4 in order to compare their induction levels. Table 4.1 shows that, among all the studied inducers only olive oil and corn oil were induced the production of the enzyme. Meanwhile, the other inducers, mainly the synthetic, did not support the growth of the bacteria and hence the enzyme production. However, olive oil was found to be the best inducer which results in 2.56 mU/ml enzyme production. In addition, no enzyme activity neither bacterial growth has been observed in the control experiment without inducer.

Due to their potential toxicity, the effect of lower concentrations (0.25, 0.5 and 0.75% w/v) of the synthetic inducers tributyrine, Tween 20 and Tween 80 on the enzyme production were studied. Table 4.2 shows that when tributyrine was supplemented to the fermentation media in 0.25, 0.5 and 0.75% w/v concentrations the bacterial growth was gradually increased with lower concentrations of tributyrine. At the same time, very low lipolytic activity (0.55 mU/ml) has been detected when 0.25 % (v/v) tributyine was used. Similar results were obtained, regarding enzyme production, with Tween 20 where the presence of 0.25% w/v of the inducer in the fermentation media were supported 0.29 mU/ml enzyme production.

On the other hand, no lipolytic activities were detected in the supernatant of the culture media when 0.25, 0.5 and 0.75% (w/v) of Tween 80 concentrations were used. From the previous results, we can conclude that the best inducer for lipase production was olive oil meanwhile corn oil supported less enzyme production. Adding the synthetic lipase inducers like tributyrine, Tween 20 and Tween 80 were suppressed not only the bacterial growth and also the enzyme production which might be accredited to the toxicity of these synthetic inducers on the bacteria.

In contrast to our results, Tweens were reported as effective inducers for lipase production by many *Acinetobacter* species. For example, lipase of *Acinetobacter radioresistens* was produced using Tween 80 as the carbon source (Li *et al*., 2001).

Table 4.1 Effect of different synthetic and natural lipase inducers on lipase production by *Acinetobacter* sp. K5b4. In this experiment all inducers were added to the growth media in 1.0% (w/v) concentration. The control experiment was the growth media without inducer. ND denoted for not detectable.

Inducer	**Lipase Production (mU/mL)**	**Growth (g/L) dry weight**
Control	ND	ND
Olive oil	2.56 ± 0.18	1.87
Corn oil	0.12± 0.1	0.87
Tributyrine	ND	0.147
Tween 20	ND	ND
Tween 80	ND	ND

In addition, the supplement of Tween 80 (1.0% v/v) to the culture media, contain olive oil emulsion, of *Acinetobacter haemolyticus* TA106 resulted in the enhancement of lipase production (Jagtap *et al*., 2010). (Gupta *et al*., 2012) in their investigation about lipase production by *Bacillus* sp. strain DVL2 in three different media found that the medium containing tributyrin and tween 80 supported the highest lipase and esterase production comparing to the other two media containing olive oil as lipase inducer.

In another study, when Tween 80 was used as inducer in the culture media of the *Acinetobacter* species strains (BK43 and BK44), both strains utilized Tween 80 and produced a great level of extracellular lipase meanwhile using tributyrin resulted in very low enzyme production by both strains (Anbu *et al*., 2011). Similar to our results, adding Tween 80 to the normal complex medium of psychrophilic and mesophilic *Acinetobacter* species inhibited lipase production (Breuil and Kushner, 1975).

Table 4.2 Effect of different concentrations of synthetic inducers on lipase production by *Acinetobacter* sp. K5b4. The control experiment was the growth media without inducer. ND denoted for not detectable.

Inducer	**Concentration (%w/v)**	**Lipase Production (mU/mL)**	**Growth (g/L) dry weight**
Tributyrin	Control	ND	ND
	0.25	0.55 ± 0.07	0.20
	0.5	ND	0.19
	0.75	ND	0.18
Tween 20	Control	ND	ND
	0.25	0.29 ± 0.03	0.02
	0.5	ND	ND
	0.75	ND	ND
Tween 80	Control	ND	ND
	0.25	ND	0.07
	0.5	ND	ND
	0.75	ND	ND

However, in another experiment, the effect of different concentrations of olive oil as lipase inducer in the fermentation media was studied. The results revealed that Figure 4.13 the maximum enzyme production (2.7 mU/mL) was recorded when olive oil was added to the culture media in 1.0% (w/v) concentration. At concentrations lower and higher than 1.0% (w/v) less amount of the enzyme was detected in the cell free supernatant. On the other hand, the bacterial growth was increased with the increase in olive oil concentration with the highest of 2.2 g/L dry weight detected when 1.5% (w/v) olive oil was used. Previously, it has been reported that oil contents higher than 1.5% led to serious oxygen transfer limitations (Lima *et al*., 2003).

However, to this point of optimization the results from the previous experiments clearly indicated that changing the studied parameters as well as the lipase inducers did not affect the magnitude of lipase production even

though it shows the optimum setting values. Our findings were support the conclusion that the presence of glucose in *Acinetobacter* sp. K5b4 growth media might led to catabolic repression on lipase production. Therefore, the following experiment was performed to test this assumption.

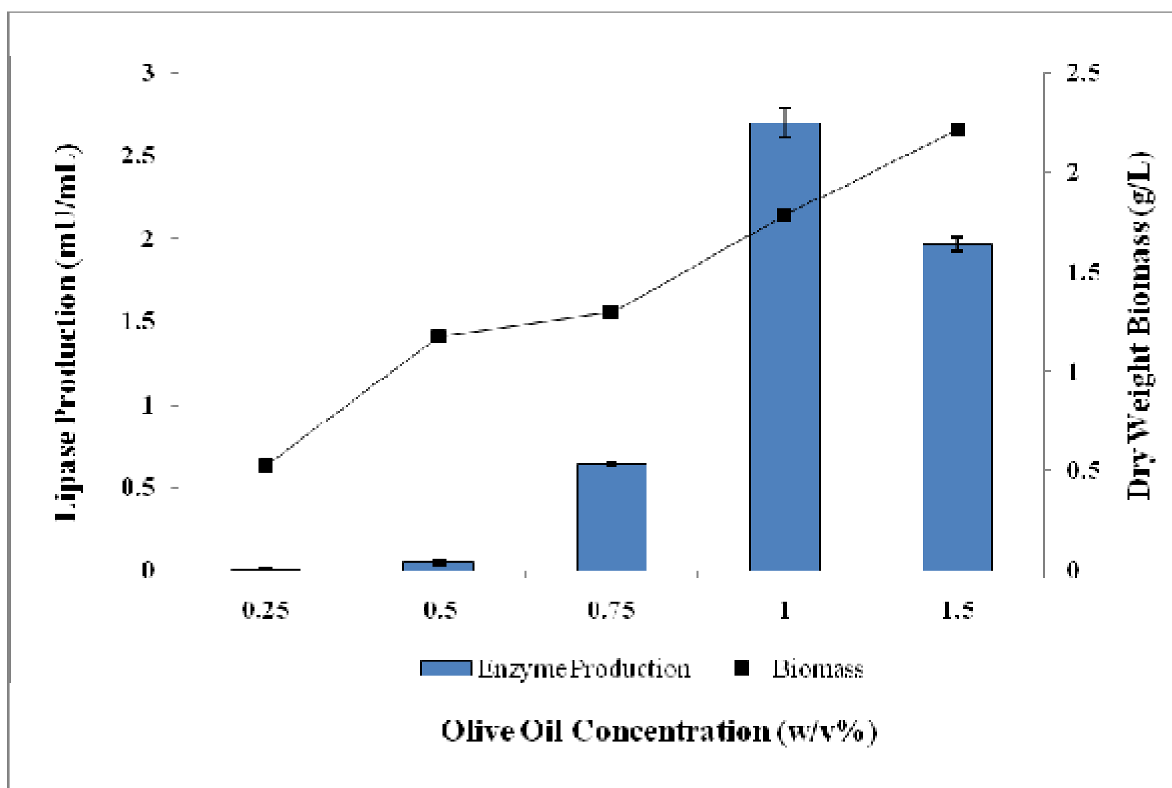


Figure 4.13 : Effect of different concentrations of olive oil in the culture media on lipase production by *Acinetobacter* sp. K5b4**.** Bars presented for lipase production are mean values of ± standard deviation of triplicates.

4.6 Effect of glucose concentration on lipase production from *Acinetobacter* sp. K5b4

Obviously, the previous results showed that changing the inoculum size, incubation temperature, pH, agitation speed and applying different inducers in the growth media of *Acinetobacter* sp. K5b4 did not affect the magnitude of lipase production even though it shows the optimum setting values of these parameters. Therefore, in this experiment our attempt to investigate the role of glucose in the catabolic repression of lipase production from *Acinetobacter* sp. K5b4. The Figure 4.14 clearly revealed that the enzyme production was increased with the decrease in glucose concentration

in the growth media. The maximum lipase production of 4.2 mU/mL was obtained in the control experiment without glucose as well as at the lowest glucose concentration tested (0.05% w/v).

On the other hand the growth of *Acinetobacter* sp. K5b4 was reduced with the decrease of glucose concentration in the fermentation media. Previous report (Jagtap *et al*., 2010) showed that the addition of glucose to the fermentation media of *Acinetobacter haemolyticus* TA106 was also resulted in sever reduction in lipase production (1.6 U/ml) comparing to the control experiment which is grown on LB and olive oil emulsion (42 U/ml). Our results and (Jagtap *et al*., 2010) findings were support the assumption that the presence of glucose in *Acinetobacter* sp. K5b4 led to catabolic repression on lipase production.

Additionally, *Burkholderia glumae* when grown on media containing lipase inducers such as olive oil, Tween 80 was found to induce lipase gene expression in strain PG1 in medium containing sucrose as a carbon source but not in glucose-containing medium, suggesting that lipase gene expression is prone to catabolic repression(Bouke *et al*., 2007). Similar level of catabolic repression on lipase production was also observed due to glucose when *Rhizopus delemar* was grown in submerged and solid state cultures (Christen *et al*., 1995).

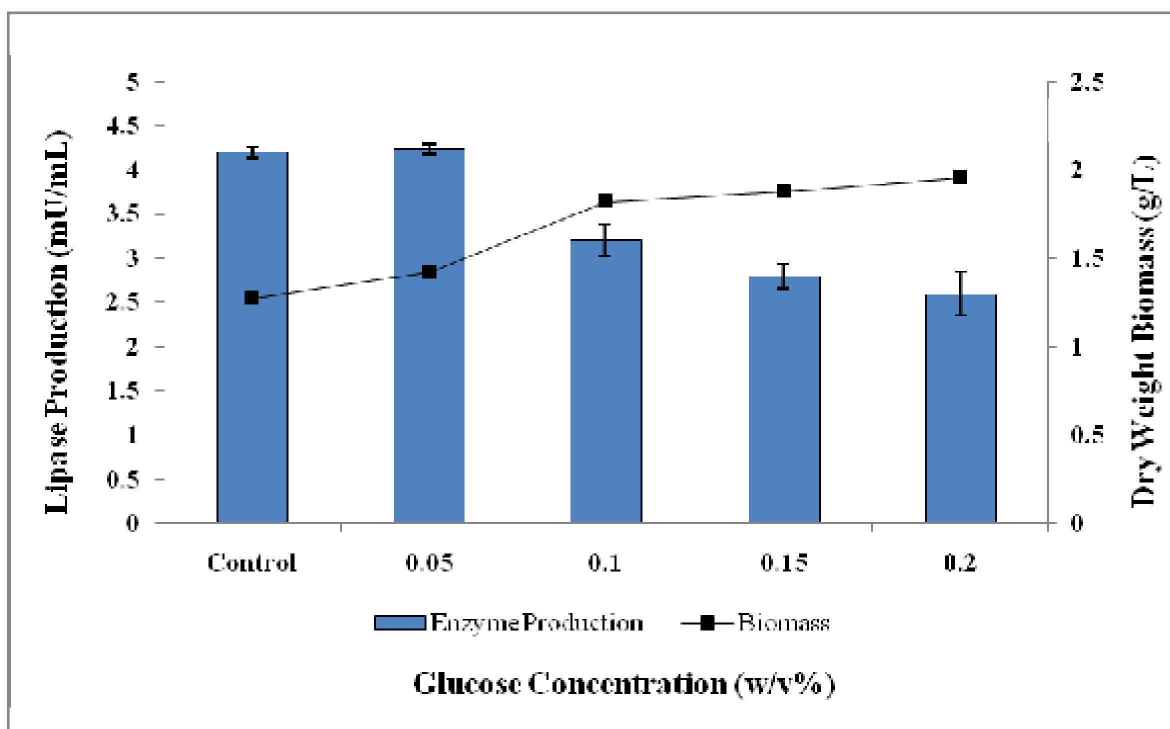


Figure 4.14 : Effect of different concentrations of glucose in the culture media on lipase production by *Acinetobacter* sp. K5b4**.** Bars presented for lipase production are mean values of ± standard deviation of triplicates.

4.7 Effect of carbon sources (other than inducers) on lipase production from *Acinetobacter* sp. K5b4

The results obtained from this experiment clearly showed that Table 4.3 most of the studied carbon sources, supplemented to the culture medium in 0.2% w/v, were supported higher enzyme production compared to the control experiment without carbon source. Maximum lipase production of 19.8 and 17.9 mU/mL was noted with glycerol and starch, respectively. This 4.7 and 4.2 folds enhancement in lipase production when glycerol and starch was supplemented to the fermentation media was not corresponding to the results of the bacterial growth. Less biomass production was recorded in the case of glycerol and starch compared to the control experiment and other carbon sources (sorbitol, mannitol and sucrose) with the highest biomass production of 4.7 g/L dry weight biomass was recorded when sorbitol was used as carbon source in the growth media. However, among the studied carbon sources maltose and lactose did not support the enzyme production even though a considerable bacterial biomass was observed in the growth media.

In another experiment, the effect of different concentrations of glycerol and starch on lipase production by *Acinetobacter* sp. K5b4 was studied. Table

4.4 and 4.5 showed that the maximum lipase production for both carbon sources of 19.4 and 17.4 mU/mL, respectively were obtained at 0.2% (w/v) of glycerol and starch. Meanwhile, adding both carbon sources to the growth media in concentrations above or below 0.2% (w/v) supported less enzyme production.

In the case of glycerol, biomass production was corresponding to the enzyme production where the maximum biomass production was also recorded at 0.2% (w/v) concentration. Meanwhile, adding glycerol to the growth media in concentrations lower and higher than 0.2% (w/v) were supported less biomass production when compared to 0.2% (w/v) concentration. However, the biomass production when different concentrations of starch was studied increased with the increase in the concentration with the highest value of 1.5 g/L recorded at 0.3 and 0.4% (w/v). However, the presence of glycerol in the fermentation media as the sole carbon source, without olive oil, resulted in very low enzyme production of 1.58 mU/mL. On the other hand, no enzyme activity was detected when starch added to the fermentation media as the sole carbon source without olive oil.

Table 4.3: Effect of different carbon sources (0.2% w/v) on lipase production by *Acinetobacter* sp. K5b4. The control experiment was the growth media without carbon source rather than olive oil. ND denote for not detectable.

Carbon Source	Lipase Production (mU/mL)	Growth (g/L) dry weight
Control	4.2 ±0.23	1.55
Starch	17.9 ±0.22	0.87
Glycerol	19.8 ±0.35	0.79
Sorbitol	6.4 ±0.32	4.77
Mannitol	7.7 ±0.2	2.58
Maltose	ND	0.71
Fructose	5.4 ±0.03	0.4
Lactose	ND	0.33
Sucrose	7.4±0.09	3.22

Table 4.4: Effect of different concentrations of glycerol on lipase enzyme production by *Acinetobacter* sp. K5b4.

Starch Concentration (w/v)%	Lipase Production (mU/mL)	Growth (g/L) Dry weight
Control	5.07 ±0.43	1.53
0.1	14.96 ±0.32	0.87
0.2	17.43±0.44	0.94
0.3	10.82±0.45	1.51
0.4	9.35±0.44	1.50
(0.2% w/v) starch w/o olive oil	**ND**	**0.01**

Table 4.5: Effect of different concentrations of starch on lipase enzyme production by *Acinetobacter* sp. K5b4

Glycerol Concentration (w/v)%	Lipase Production (mU/mL)	Growth (g/L) Dry weight
Control	5.07 ±0.43	1.47
0.1	12.82 ±0.72	0.69
0.2	19.38±0.15	0.82
0.3	17.84 ±0.19	0.54
0.4	9.21±0.42	0.37
(0.2% w/v) glycerol w/o olive oil	**1.58±0.44**	**0.11**

The findings reviewed from previous reports regarding the effect of the presence of additional carbon sources in addition to the enzyme inducer generally have appositive effect on lipase production by different species of *Acinetobacter* as compared to the media with the inducer alone. For instance, it was reported that the presence of sucrose in the fermentation media of *Acinetobacter haemolyticus* together with olive oil has positive effect on lipase production compared to the media with olive oil alone (Jagtap *et al*.,

2010). In agreement with (Jagtap *et al*. 2010) results the effects of different carbon sources on lipase production by two strains of *Acinetobacter* (BK43 and BK44) were evaluated. The greatest increase in lipase production by both strains was observed in response to supplementation of the culture medium with sucrose as the carbon source, followed by lactose in the case of BK43and xylose in the case of BK44 compared with glucose (control). The other carbon sources evaluated, such as fructose, starch and mannitol, led to a decrease in lipase production by BK44 of more than 50%. Their results suggest that the above carbon sources act as inhibitors of lipase production and indicate that the carbon source has the potential to increase the lipase production significantly (Anbu *et al*., 2011).

4.8 Effect of nitrogen sources on lipase production from *Acinetobacter* sp. K5b4.

In this experiment, different nitrogen sources were evaluated for their effects on the extracellular production of lipase enzyme from the bacterial strain *Acinetobacter* K5b4. The results in Figure 4.15 shows that, the presence of nitrogen sources in the growth media have a critical effect on lipase production from *Acinetobacter* sp. K5b4 as 0.2 g/L biomass production with no lipase activity was detected in the control experiment. On the other hand, adding ammonium chloride, tryptone, peptone and ammonium nitrate to the growth media resulted in comparable enzyme activity of 19.4, 20.5, 21.1 and 19.1 mU/mL, respectively. Less enzyme production was observed when urea and sodium nitrate were used as the sole nitrogen sources in the growth media with enzyme production of 17.1 and 16.5 mU/mL, respectively. However, the maximum enzyme production as well as biomass yield of 27.7 mU/mL and 3.5 g/L was obtained when 0.1% (w/v) yeast extract was used.

For further investigation of the effect of yeast extract on the extracellular lipase production, different concentrations of yeast extract were added to the growth media and the enzyme and biomass production were evaluated. Figure 4.16 shows that maximum lipase production of 28.9 mU/mL of enzyme activity was detected when 0.15% (w/v) yeast extract was supplemented to the growth media. The biomass production was in accordance to the enzyme production in that the highest biomass production of 3.7 g/L was obtained when 0.15% (w/v) yeast extract was supplemented to the growth media. When yeast extract was used in concentrations lower and higher than the optimum concentration, less enzyme and biomass production was recorded.

Several research articles were documented yeast extract as the best nitrogen source to support the highest lipase production by different

microorganisms. For instance,(Kumar *et al*. 2012) reported that peptone and yeast extract as nitrogen sources in the growth media supported the highest lipase production by various *Bacillus* sp. and *Pseudomonads* sp. In another study, the results showed that yeast extract produced maximum lipase activity, from *Penicillium citrinum* followed by peptone, tryptone and beef extract (Hasan *et al*., 2001). The effect of nitrogen source on lipase production by *Bacillus licheniformis* indicated that the yeast extract was the suitable substrate for accelerating lipase production (Larbidaouadi□s *et al*., 2014).

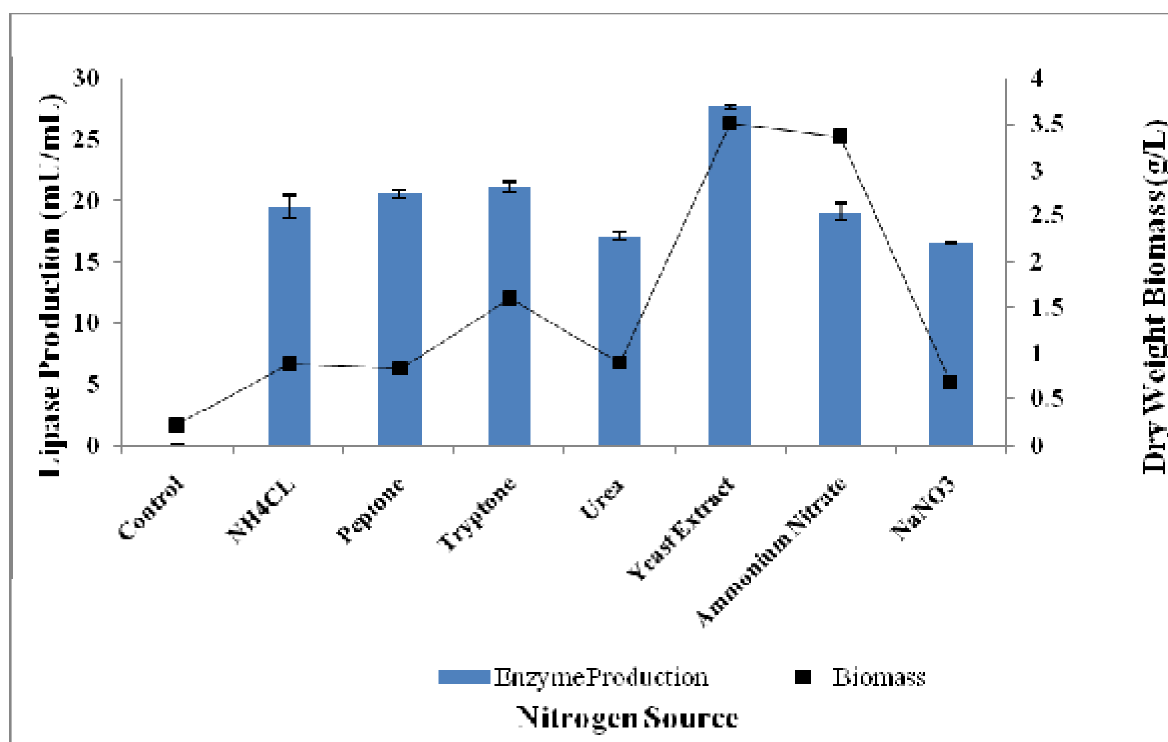


Figure 4.15: Effect of different nitrogen sources on lipase production by *Acinetobacter* sp. K5b4.

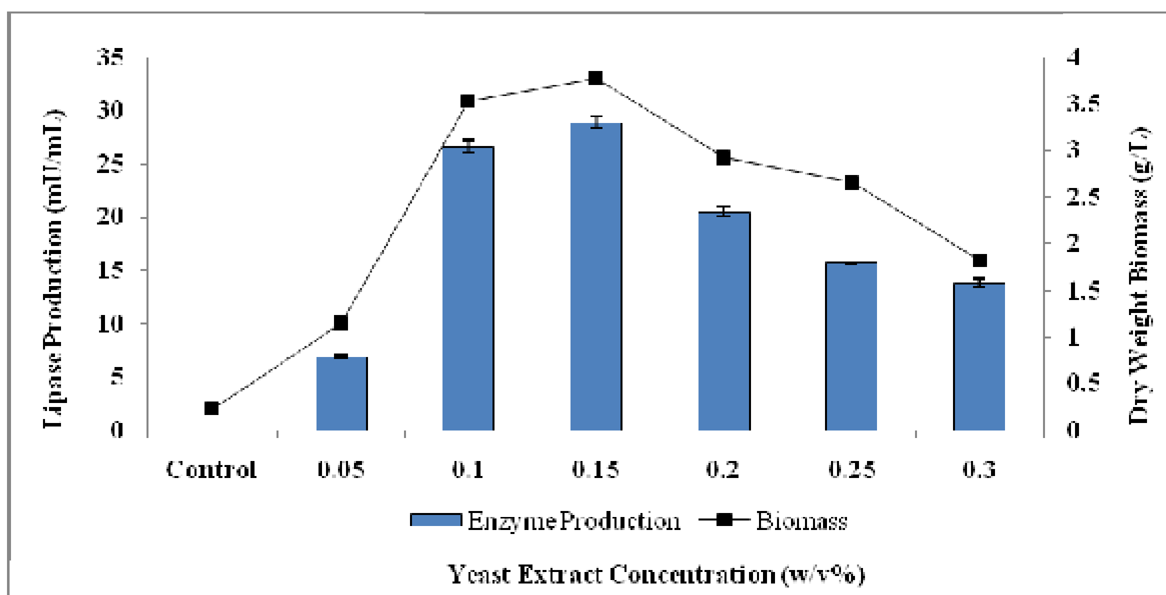


Figure 4.16: Effect of different concentrations of yeast extract on lipase production by *Acinetobacter* sp. K5b4.

4.9 Effects of mineral salts and NaCl concentration on lipase production from *Acinetobacter* sp. K5b4.

In this experiment the role of various micronutrients on the extracellular lipase production were evaluated. The results in Figure 4.17 revealed that the tested mineral salts were did not enhance the enzyme production as it compared to the control experiment prepared without addition of any mineral salts. However, the presence of $CaCl_2$ in the growth media did not show any inhibitory or stimulatory effect on the enzyme production as approximately similar results of lipase and biomass production was recorded compared to the control experiment. In contrast to the results of $CaCl_2$, the other mineral salts ($MgCl_2$, $MnCl_2$, KCl and $CoCl_2$) that tested in this experiment were negatively affected the production of lipase enzyme. For instance, when $MgCl_2$ and $CoCl_2$ were added to the fermentation media no enzyme activity was detected even though high biomass productions were observed and even more than the control experiment when $MgCl_2$ was used. $MnCl_2$ and KCl, on the other hand, caused reduction in the enzyme production of 96 and 85%, respectively. Similar results were obtained with *Pseudomonas aeruginosa* MTCC 10,055 where the addition of various metal salts resulted in reduction of lipase production compared to the control experiment containing $MgSO_4$ with no lipase activity detected when Cu SO_4 was used (Bisht *et al*., 2012). In contrast to these results, (Dhiman *et al*. 2013) reported a stimulatory effect by Ca^{2+} alone or in combination with other ions such as Mg^{2+} and Fe^{2+}on lipase

production by strains of *Bacillus*. In addition, Iron was found to play a critical role in the production of lipase by *Pseudomonas* sp. G6 (Sugihara *et al*. 1991)

The bulk ions, such as Na^+, K^+, and $C1^-$ reported to have physiologic functions associated with their osmotic effects and influence onmacromolecular behavior (Stubblefield and mueller, 1960). However, the role of NaCl on lipase production by *Acinetobacter* sp. K5b4 was investigated. In Figure 4.18, the results showed that the enzyme and biomass production was increased with the increase in NaCl concentration with the maximum enzyme and biomass production of 31.5 mU/mL and 3.3 g/L were obtained with 0.05% (w/v). Gradual decreases in the enzyme and biomass production were observed with the elevated concentrations of NaCl. Similar results by (Jagtap *et al*. 2010) have been reported when they studied the effect of NaCl on lipase production by *Acinetobacter haemolyticus* AT106 where they found that the presence of suitable concentration of NaCl in the growth media enhanced the enzyme production. They also reported that, the increases in NaCl concentration were caused reduction in the enzyme production.

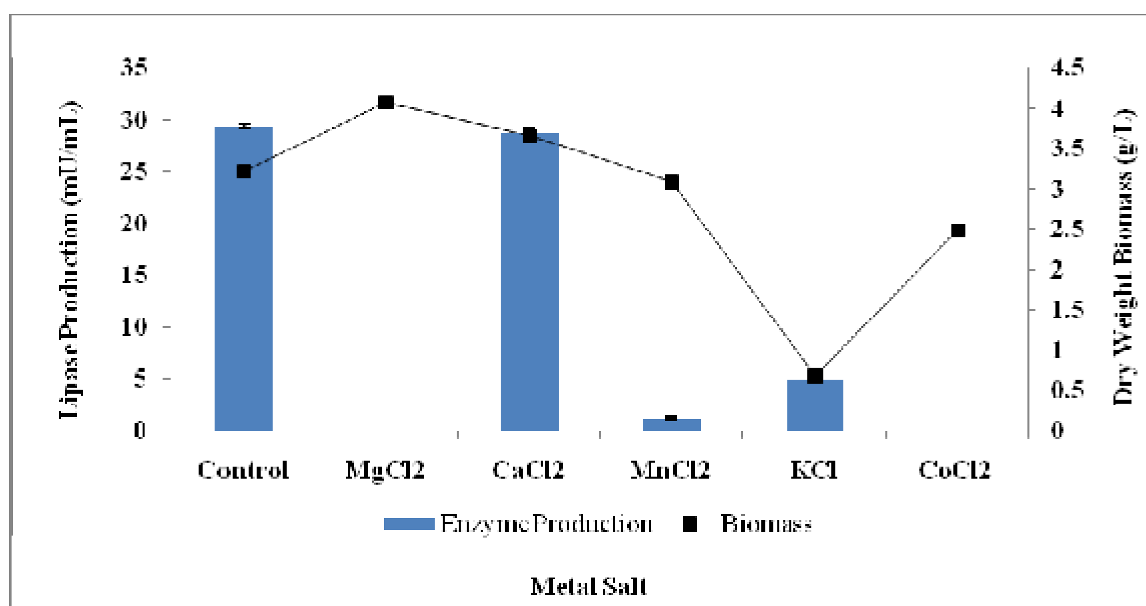


Figure 4.17 : Effect of different metal salts on lipase production by *Acinetobacter* sp. K5b4.

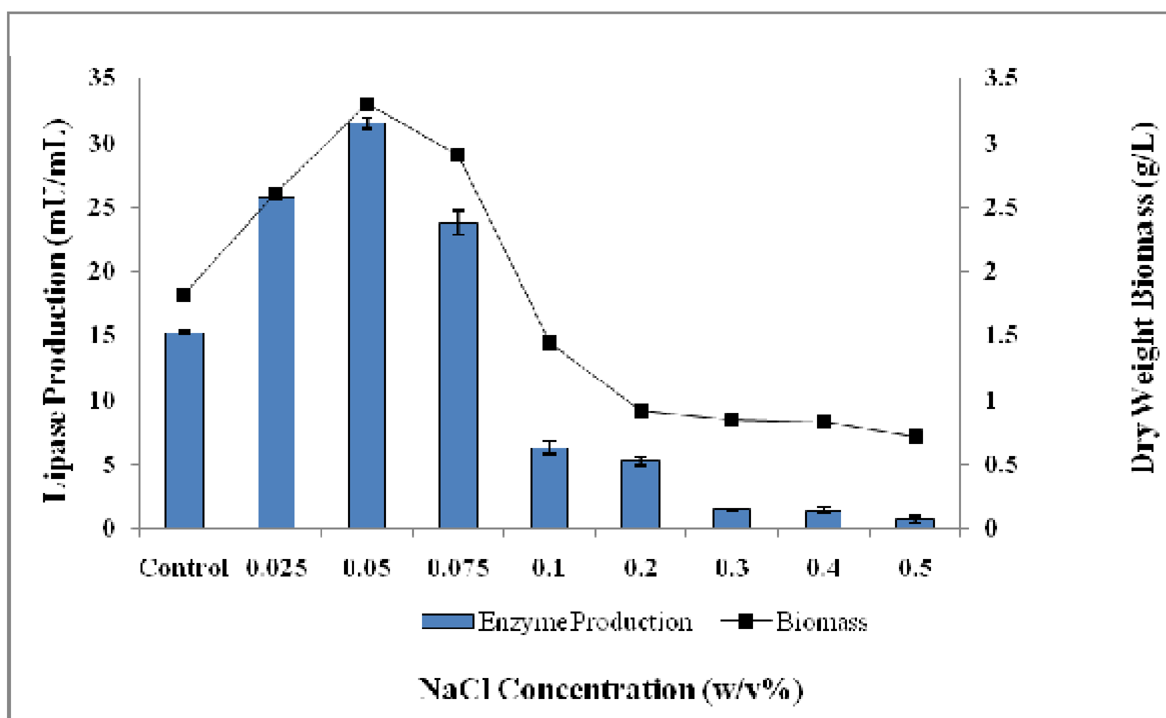


Figure 4.18 : Effect of different concentrations of NaCl on lipase production by *Acinetobacter* sp. K5b4.

4.10 Conclusion and Recommendations.

The screening experiment for lipase producing bacteria from 13 soil samples contaminated with hydrocarbons resulted in the isolation of 34 bacterial isolates with extracellular lipolytic activity. The stability of the extracellular lipases produced by these isolates was further investigated against methanol and ethanol organic solvents. Out of the 34 isolates, 7 bacterial isolates namely K9a3, K9a2, K7a1, K9a1, K7a3, K5b4 and K7b2 were selected based on the results of the stability experiment of their lipases after it has been exposed to the hydrophilic organic solvents, methanol and ethanol. Among the 7 isolates, the extracellular lipase produced by the bacterial strain K5b4 shows the highest stability in the presence of both organic solvents in the assay solutions. This bacterial isolate which identified as *Acinetobacter* sp. was selected for further studies.

The physicochemical optimization of lipase production by *Acinetobacter* sp.K5b4 in shake flask system showed that, maximum lipase activity of 31.5 mU/mL in the culture supernatant was obtained after 48 hr cultivation when the fermentation medium (pH 7.0) composed of 1.0% (w/v) olive oil, 0.2% (w/v) glycerol, 0.15% (w/v) yeast extract, and 0.05% (w/v) NaCl was inoculated with 0.4% (v/v) seed culture and incubated at 30°C and

150 rpm agitation speed. However, all the tested mineral salts were did not enhance the enzyme production as it compared to the control experiment. The results clearly revealed that the presence of glucose in the culture medium caused catabolic repression on lipase production.

Further research studies can be attempted by taking advantage of the data obtained in the present work. Generally, such a future research may involve the following outlines.

1. Further studies were highly recommended to expand the characterization work of the enzyme that helps in better understanding of the enzyme properties
2. Application of *Acinetobacter* sp. K5b4 lipase enzyme in the biosynthesis of biodiesel and other optically pure fine chemicals.

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Appendix I

RapID ONE Report

Thermo SCIENTIFIC

ERIC™ *Electronic RapID Compendium*

Laboratory: My Laboratory
User: MyLogon

Ref No: 14.0000001
Report Date: 11/25/2014

RapID ONE

Identification Report

Microcode: 0400000

- URE	- LDC	- KSF	- ONPG	- NAG	- GGT	- IND
- ADH	- TET	- SBL	- βGLU	- MAL	- PYR	- OXI
- ODC	+ LIP	- GUR	- βXYL	- PRO	- ADON	

IDENTIFICATION = Acin. calcoaceticus

Choice(s)	Probability	Bioscore	Contraindicated Tests
Acin. calcoaceticus	**>99.9%**	**1/3**	**None**

Probability Level: Implicit   BioFrequency: Typical

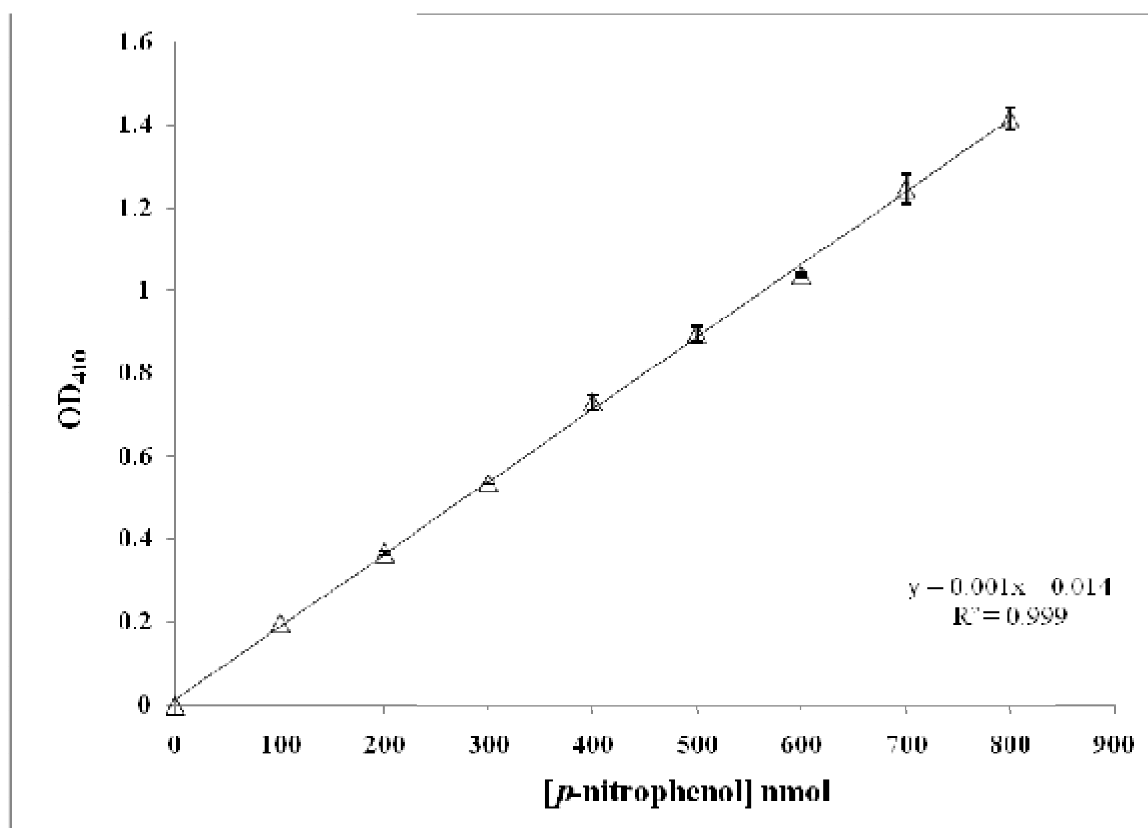
Free living and widely distributed in nature and the hospital environment. Found in a variety of clinical specimens. Species definition within Acinetobacter is uncertain. There are 17 genospecies defined.

Version: 1.1.0
Remel Inc. Lenexa, Kansas

Printed on: Tuesday November 25 2014

Appendix II

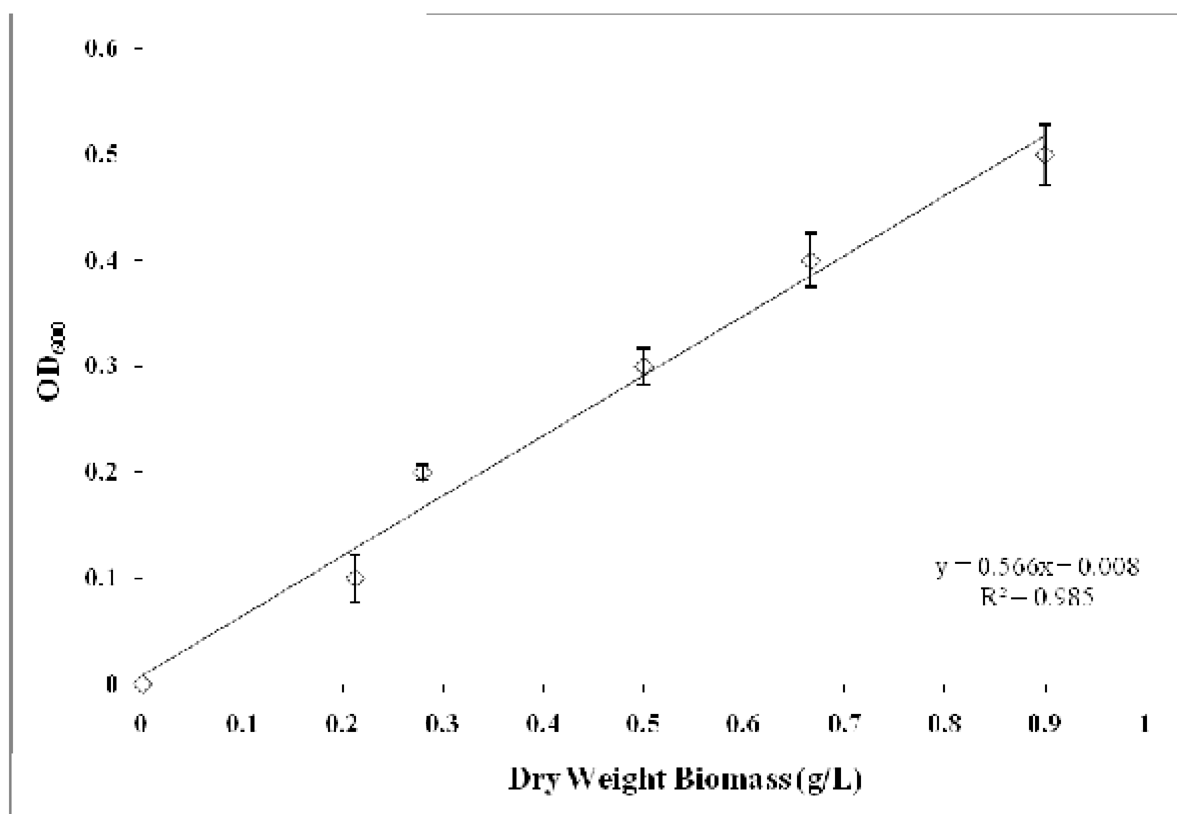
p-nitrophenol standard curve



1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD_{410}
y – 0.001x 0.014
R^2 = 0.999
0
100
200
300
400
500
600
700
800
900
[*p*-nitrophenol] nmol

Appendix III

Dry Weight Biomass Standard Curve


0.6
0.5
0.4
0.3
0.2
0.1
0
OD_{600}
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
Dry Weight Biomass (g/L)
y = 0.566x - 0.008
R^2 = 0.985

المعلومات الشخصية

الاسم: سناء راتب العنانزة

التخصص: ماجستير العلوم الحياتية

الكلية: العلوم

السنة: 2014

هاتف رقم: 0775510850